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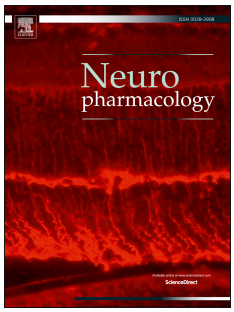
Published in:
Neuropharmacology

DOI:
10.1016/j.neuropharm.2017.07.005

Publication date:
2017

Accepted Manuscript



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PII: S0028-3908(17)30312-X

DOI: 10.1016/j.neuropharm.2017.07.005

Reference: NP 6774

To appear in: *Neuropharmacology*

Received Date: 11 February 2017

Revised Date: 4 July 2017

Accepted Date: 6 July 2017

Please cite this article as: Gacsályi, Istvá., Móricz, K., Gigler, Gá., Wellmann, Já., Nagy, K., Ling, Istvá., Barkóczy, Jó., Haller, Jó., Lambert, J.J., Szénási, Gá., Spedding, M., Antoni, F.A., Behavioural pharmacology of the α5-GABA$_A$ receptor antagonist S44819: Enhancement and remediation of cognitive performance in preclinical models, *Neuropharmacology* (2017), doi: 10.1016/j.neuropharm.2017.07.005.

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ABSTRACT

Previous work has shown that S44819 is a novel $GABA_A$ receptor ($GABA_AR$) antagonist, which is selective for extrasynaptic $GABA_ARs$ incorporating the α5 subunit (α5-$GABA_ARs$). The present study reports on the preclinical neuropsychopharmacological profile of S44819. Significantly, no sedative or pro-convulsive side effects of S44819 were found at doses up to 30 mg/kg *i.p.*. Object recognition (OR) memory in intact mice was enhanced by S44819 (0.3 mg/kg *p.o.*) given before the acquisition trial. Mice treated with phencyclidine for two weeks and tested six days after the cessation of treatment failed to show OR memory. This deficit was corrected by a single administration of S44819 (0.1, 0.3 or 1 mg/kg *p.o.*) prior to the acquisition trial. The amnestic effect of ketamine in rats tested in the eight-arm radial maze (reference and working memory versions) was blocked by S44819 (3 mg/kg *p.o.*). Extinction of cued fear was preserved during treatment with S44819 (3 mg/kg/*diem i.p.*). Administration of S44819 had no significant effect in the Vogel-conflict test, the elevated plus maze, the forced swim, the marble-burying and the tail-suspension tests. In contrast, anxiolytic/antidepressant-like effects of the compound were found in paradigms that have mnemonic components, such as social interaction, fear-potentiated startle and social avoidance induced by negative life experience. In summary, S44819 enhanced intact recognition memory and ameliorated memory deficits induced by inhibition of NMDA receptors. Anxiolytic/antidepressant efficacy was limited to paradigms involving cognitive function. In conclusion, S44819 is a novel psychoactive pro-cognitive compound with potential as a therapeutic agent in dementia.

1. Introduction

Gamma-aminobutyric acid (GABA) is a fundamentally important neurotransmitter in the CNS (Klausberger and Somogyi, 2008; Walker, 1983). The actions of GABA are mediated by ionotropic $GABA_A$ ($GABA_A$Rs) and metabotropic $GABA_B$ receptors. The topographical distribution of $GABA_A$R subunits in the brain indicates non-redundant functions (Pirker et al., 2000; Wisden et al., 1992). Indeed, in a pioneering studies analysing genetically engineered mice, the groups of Rudolph and Möhler and of McKernan and Whiting (reviewed by (Rudolph and Möhler, 2014)), showed that the diverse effects of diazepam on brain function were attributable to $GABA_A$Rs incorporating specific α-subunits. These studies were the first to show that α5-$GABA_A$Rs influence cognitive functions. Thus, the physiologic rationale of producing $GABA_A$ isoform selective pharmacons as therapeutic agents was established (Dawson et al., 2005).

The chemical synthesis and biological characterization of a family of tricyclic compounds, based on a 2,3-benzodiazepine scaffold, that are competitive inhibitors of GABA binding at the α–β subunit interface, was previously reported (Etherington et al., 2017; Ling et al., 2015). For some compounds, orthosteric antagonism was paired with inhibition of Cl^--channel gating, similar to the properties of the $GABA_A$R antagonist, bicuculline (Ueno et al., 1997). However, in contrast to bicuculline, which is a non-selective blocker of most $GABA_A$R isoforms, some of the novel compounds show considerable isoform selectivity. A potentially valuable compound in the series is S44819, which is a largely selective inhibitor of extrasynaptic α5-$GABA_A$Rs *in vitro* (Etherington et al., 2017). The current paper reports the *in vivo* preclinical pharmacology of this molecule. The results show enhancement of normal as well as remediation of impaired cognitive function, including object recognition memory, spatial working and reference memory by S44819. The compound also showed significant anxiolytic/antidepressant-like activity in tests that have a mnemonic component.

2. Methods

2.1 Animals

Male rats (190–270 g BW), or mice (20-30 g BW) — the strains are specified under the headings of the respective tests — were housed under standardized laboratory conditions (24 ± 2 ºC, 40–60% relative humidity), on a 12-h light/dark cycle with light onset at 6:00 AM. All experimental protocols were approved by the Animal Care and Use Ethical Committee of Egis Pharmaceuticals PLC and complied with the Hungarian Law of Animal Care and Use (1998. XVIII).

2.2 Compounds

S44189 (Egis-13529), substituted *8-methyl*-5-[*1-benzothiophen-2-yl*]-*1*,*9-dihydro-2H-[1*,*3*]*oxazolo* [*4*,*5-h][2*,*3*]*benzodiazepin-2-one* was produced as free base at greater than 97% purity at Egis Pharmaceuticals PLC as previously described (Ling et al., 2012; Ling et al., 2015). The solubility of the compound in physiological buffers containing 0.1% DMSO (v/v) was around 50 µM. Furthermore, because of the poor oral bioavailability of S44819 (F < 2%) the compound had to be micro-formulated for preclinical studies. Two formulations of S44819 were provided by Dr Caroline Chemin, Technologie Servier, (Orleans, France). The first, Aqoat, was a suspension of micro-milled S44819/Hypromellose Acetate Succinate (Shin Etsu Chemical Co, Japan)/Magnesium stearate (30/69.5/0.5, w/w/w) suspended in 2% (w/v) hydroxy-ethylcellulose (Molar Chemicals Ltd, Budapest). The second, (nanoF3) was a nano-encapsulated formulation consisting of S44819 3.85/Lipoïd S100 (Lipoïd GmbH, Ludwigshafen, Germany) 38.5/trehalose 57.7, w/w/w). Both formulations were used for intra-peritoneal (i.p.) as well as oral (p.o.) administration. The preparations were administered on the basis of their free base active product ingredient content. An effort was made to minimize the exposure of the animals to the excipients. Thus, the amount of excipient used depended on the highest dose of the drug administered in a given experiment — importantly, the dose of excipient was kept constant within a particular study. The highest dose of S44819 formulated with Aqoat was 30 mg/kg, thus all animals in received 0.50 mg/kg magnesium

stearate, 71 mg/kg hypromellose acetate succinate and 0.1 mg/kg of hydroxy-ethylcellulose. The dose of excipients was kept constant with reduced doses of drug. In the case of nanoF3, the highest dose used was 30 mg/kg of S44819, thus the animals also received 300 mg/kg Lipoïd S100 and 450mg/kg of trehalose. The dose of excipients was kept constant with reduced doses of drug. The drug administration regimes described below were optimized on the basis of preliminary kinetic studies in mice and rats with both formulations of S44819. Notably, both formulations gave similar brain:plasma concentration ratios approximating 1:2 in male mice (see Suppl. Fig.-s 1 and 2) and 1:4 in male rats (data not shown).

2.3 Object recognition assay in mice

Male albino NMRI mice bred in house were used. The experimental protocol was analogous to that described for rats (Gacsályi et al., 2013). Briefly, Day 0, familiarization: mice spent 2.5 minutes in a 24 x 34 x 24 cm box with black plastic walls. Acquisition: on the next day mice were allowed to explore freely two identical objects placed symmetrically at 4-4 cm from the shorter sides of the box. The animals were removed from the box when exploration times of both objects reached 10 s/object during a maximum 5 min period. Retention: 24 h later the animals were allowed to explore a novel object and an identical copy of the familiar object — the exploration time for both objects was recorded for 4 min. Treatment with Aqoat-formulated S44819 (0.03, 0.1, 0.3 mg/kg), or vehicle was performed *p.o.* 120 min before the start of the acquisition trial. Data were analysed by one-way analysis of variance followed by Dunnett's multiple comparison test.

In another series of experiments NMRI mice were subjected to sub-chronic treatment with vehicle (0.9% w/v NaCl), or phencyclidine (PCP, supplied by Tocris, U.K.): 3 mg/kg *i.p.* twice a day for a total of ten days (2x 5 days with a two-day break). Six days after the last PCP treatment, S44819 at 0.1, 0.3 and 1 mg/kg was administered *p.o.* 120 min before the start of the acquisition trial. The study protocol was exactly the same as above, except that the retention trial took place 15 min after the start of the acquisition period. Animals were returned to their home cage between the acquisition and retention trials. Data were analysed by Student’s t-test to validate the effect of PCP

(control vs. vehicle i.e. 0 mg/kg S44819-group) and one-way ANOVA followed by Dunnett's multiple comparison test (vehicle *vs.* S44819-treated groups) to assess the effect of S44819.

2.4 Working memory in the eight-arm radial maze in rats

Male Lister-Hooded rats 185-228 g body weight (Harlan Laboratories, The Netherlands) were trained and tested in an eight-arm-maze-based spatial working memory task in a dimly lit special room where geometric shapes were placed on the walls as visual cues. (Gacsályi et al., 2013). The maze was constructed from Perspex. It consisted of a central octagonal platform (diameter: 28 cm) and 8 radial direction arms (width: 9.5 cm, length: 70 cm, wall height: 12 cm). The central part and the arms had removable Perspex covers. Bait-food (5 mm diameter pellets made from a mixture (1:1, w/w) of ground tea-biscuits (Győri Otthon Keksz, Tesco stores, Budapest) and instant cocoa powder (Nesquik, Nestlé, Tesco stores)) was placed in a cavity at end of the arms. It was verified in previous independent studies, that the animals used the visual cues to find the bait**.** Young adult male Lister-Hooded rats were handled daily by the experimenter and were relatively food-deprived receiving approximately 8-10 g standard rodent food/day/animal per day. Training continued for 13 days (suspended on weekends). Rats were placed onto the central platform of the maze with all 8 arms baited. Rats were allowed to eat the bait from all 8 arms. If the rat did not eat all of the baits within 20 min it was removed from the maze. The following parameters were recorded. Repetition error (RE) is visiting the same arm more than once, Initial correct responses (ICR) is number of correct entries until first error. Animals included in the study had RE values <3 or ICR ≥7. On the last day of the experiment (after 13 days training) animals were given vehicle, or Aqoat-formulated S44819 *p.o*. (0.3, 1 and 3 mg/kg) and ketamine 10 mg/kg *i.p.*, at 120 and 30 min before the start of the trial, respectively. A single trial lasted a maximum of 5 min. The numbers of RE-s were analyzed by non-parametric Mann-Whitney test (intact control vs. ketamine control group) and Kruskal-Wallis test, followed by Dunn's multiple comparison test (ketamine control group vs. ketamine + different S44819 doses)

2.5 Reference memory in the eight-arm radial maze in rats

Reference memory was studied in male Lister-Hooded rats 230-310 g body weight with the same equipment and protocol as working memory (described above) with the following modifications. Only four arms out of eight were baited in the training sessions. Baiting was arranged in ten different configurations to the eight arms, a given animal was trained on the same configuration of arms throughout the entire experiment. Rats were allowed to eat the bait from all 4 arms. If the rat did not consume all the baits within 20 min it was removed from the maze. Training runs were performed once a day for 18 days (excluding weekends). On the last day of the experiment (after 18 days training) the animals were given vehicle, or Aqoat-formulated S44819 (0.3, 1 and 3 mg/kg, *p.o.)* and ketamine 10 mg/kg *i.p.*, at 120 and 30 min before the start of the trial, respectively. The numbers of errors, i.e. the number of entries into arms that had never been baited and the number of the missed baited arms within the 5 min of testing, were recorded. Inclusion criteria: **1)** the rat performs perfectly at least once in the last 3 learning days within 5 min, or **2**) the rat has less than 3 errors, or makes a mistake after the 3 correct entries in the last learning day in 5 or less minutes. The numbers of errors were analyzed by the Mann-Whitney U-test and the Kruskal-Wallis ANOVA followed by Dunn's multiple comparison test.

2.6 Extinction of cued fear

The subjects were male young adult C57BL/6J mice (20-25 g BW, Charles River, Germany). Apparatus: TSE Fear Conditioning System (TSE System GmbH, Bad Homburg, Germany), four animals were tested simultaneously. The protocol is summarized in Fig.5. Fear conditioning (Day 1): Mice were placed into the test chambers (21.5 cm x 21.5 cm x 35 cm) and after 3 min habituation were given the first of 3 noise/foot-shock pairings. The 0.7 mA, 2 s electric shock was delivered during the last 2 s of the 30 s exposure to white noise (85 dB). The average inter-trial interval was 2 min. Extinction procedure (Days 2 - 5): Twenty four h after the last trial, S44819 (3 mg/kg, nanoF3 formulated), or vehicle was administered *i.p*. each day 30 min before the start of

the extinction session. Mice were then placed into the same chamber where they received the conditioning and after 3 min habituation, were exposed to 4 x 30 s of white noise (85 dB) – inter-trial intervals were 60 s. The number of freezing reactions was recorded. The reaction was considered freezing if the mouse was immobile for a minimum 2 s. Freezing was scored by dividing the 30 s noise stimulus into 5 s bins. If the animal froze in all 6 bins it received a score of 6. Four noise stimuli were given in total and the percentage of bins in which freezing was evident was calculated for each animal. Data were analyzed by one-way ANOVA followed by Dunnett's test for multiple comparisons.

2.7 Fear potentiated startle response test in rats

Male Sprague-Dawley rats bred in house were used. The fear potentiated startle procedure (Davis et al., 1993; Walker and Davis, 1997) also see Suppl. Fig. 11, consisted of learning, pre-test and test periods. In the learning period over two consecutive days, male Sprague-Dawley rats were placed into the test chambers and 5 min later given the first of 10 light/foot-shock pairings. The 1 mA, 0.5 s shock was delivered during the last 0.5 s of the 3.7 s light pulse. The average inter-trial interval (ITI) was 3 min. Background noise: 55 dB. Twenty-four h after the learning period, rats were returned to the test chambers for the pre-test phase. Five min later 10 x 95 dB noise bursts (ITI: 20-40 s) were presented (habituation). After 30 s, 3 noise alone stimuli (95 dB) and 3 noise + light stimuli were presented. On the test day (24 h after the completion of pre-test) the rats were returned to the test chambers and 5 min later were presented with the same initial habituation noise. 30 s later 10-10 noise alone (95 dB) and noise + light stimuli in random order were presented to the animal. The calculated parameter is the difference in the magnitude of startle responses between "light + noise" and "noise alone" trials. (Difference = magnitude of startle response at "light-noise" trials – magnitude of startle response at "noise alone" trials). Acute treatment with vehicle, or S44819 (nanoF3, 1, 3 and 10 mg/kg *i.p.*), was on the test day 30 min before the trial. The positive control diazepam was administered acutely at 3 mg/kg, i.p. 30 min before the test. Data were analyzed by one-way ANOVA followed by Dunnett's test for multiple comparisons.

2.8 Social interaction in rats

The study was carried out on 60 male Wistar rats (Charles River Laboratories, Hungary) 190-210 g body weight. After their arrival in the animal facility, rats were housed in groups of four in cages measuring 45 x 30 x 20 cm. One week of habituation to local conditions was allowed. Rats were familiarized with the test apparatus by being placed individually into the test arena for 10 min on the last two days of the habituation. Rats were weighed on the last habituation day. Experiments were performed in two sessions on two consecutive days. Treatments were balanced over sessions. Pharmacological treatments were performed one day after the last individual exposure to the test arena, during the first half of the dark phase. The effects of vehicle and S44819 (nanoF3, 1 ,3 10 mg/kg *i.p.*) were compared together with 10 mg/kg chlordiazepoxide (CDP) as reference. Sample size was 12, but one pair was discarded from the chlordiazepoxide group for technical reasons. Therefore, the sample size was 10 in this group. Pairs of rats were placed in the test arena (60 x 50 x 40 cm) 30 min after drug treatment and observed for 10 min. The members of the pairs were unfamiliar to each other and received the same pharmacological treatment. Behavior was video-recorded through the transparent front wall of the test cage and later analyzed by means of a computer-based event recorder by an experimenter blind to the drug treatment regimen. The social interaction test arena was a dark grey plastic box of 40 x 60 x 50 cm with wood shaving bedding. Boxes were lit by white light. The front wall of the box was of transparent plastic. Six boxes were used in parallel. Fresh bedding was used for each pair of rats. The duration and the frequency of social interactions (sniffing partner; anogenital sniffing; following; allogrooming) and total amount time spent moving around the arena (exploration) were recorded. An increase in social interactions is consistent with an anxiolytic action. Increased resting and decreased exploration were considered signs of sedative or locomotor suppressive effects. The parameter analysed was the amount of time engaged in social interaction expressed

as a percentage of the total exploration time. Data were analyzed by Kruskal-Wallis ANOVA. The Mann-Whitney test was used for *post-hoc* comparisons.

2.9 Social avoidance induced by electric shock

The test was carried out in young male Wistar rats (7-9 weeks old, 250-350 g body weight) as previously reported (Leveleki et al., 2006; Sziray et al., 2007). Briefly, inescapable electric shocks (24 shocks, 3 mA, 1 s duration, 29 s interval) were delivered 1 day before the social avoidance test *via* the grid floor. Twenty-four h later, the social avoidance testing was performed in a different box from the one in which the foot shocks were delivered. Animals were injected *i.p.* with S44819 (nanoF3 0.3 and 1 mg/kg), or vehicle 30 min before the test. The subject was placed in a cage (16 x 52 cm) for a 3 min habituation period upon which a sliding door leading to a larger cage (40.5 x 41 cm) was opened. The larger cage was divided into two equal (40.5 cm x 20.5 cm) compartments by a transparent, perforated plastic wall running down the middle. The distant compartment contained a large, unfamiliar male Wistar rat (>11 weeks old, >400 g body weight), as the opponent. The opponent was un-shocked. The subject was allowed to explore the resulting space for 5 min. The test apparatus did not permit physical contact between the experimental and stimulus animals. The duration of visits made to the middle compartment (opponent time) was recorded and expressed as percentage of the total time of exploration (% opponent time). Data were analyzed by one-way ANOVA followed by Dunnett's test for multiple comparisons.

3. Results

Analysis of spontaneous locomotor activity (see Supplementary methods) indicated that nano-formulated S44819 had no sedative, or anxiogenic actions at doses up to 30 mg/kg *i.p.* (Fig. 1A). Further tests of anxiety were in agreement with this conclusion (Suppl. Figs. 4-7). Moreover, there were no signs of muscle weakness/relaxation in the inclined screen test at doses up to 100 mg/kg in marked contrast with Baclofen and Diazepam at 10ml/kg, *i.p.* both of which had a significant effect on muscle strength (Suppl. Fig. 3). Non-selective inhibition of $GABA_A$ receptors results in the lowering of the convulsion threshold (Stephens and Turski, 1993). This was indeed the case upon chronic administration of pentylenetetrazole (40 mg/kg *i.p,* see Supplementary methods): by day 18, 60 % of the animals displayed clonic seizures (Fig.1B). In contrast, there were no signs of seizures in the group given the same dosage of S44819 (Fig.1B).

3.1 Novel object preference in mice

In intact male mice, acute administration of Aqoat formulated S44819 at 0.3 mg/kg *p.o.,* given 120 min prior to the acquisition trial, significantly increased the time spent investigating the novel object over the familiar object 24 h after acquisition (Fig. 2). Note that in this study the time for object exploration and the inter-trial interval were adjusted so that the control animals had no significant retention of the object explored during the acquisition trial. Other dosage regimes of the drug were not tested.

The effect of S44819 on PCP-induced memory deficit was also examined. As reported previously by others (Grayson et al., 2007; Redrobe et al., 2010), mice subjected to sub-chronic treatment with PCP failed to show significant short-term object recognition memory (Fig. 3). Acute administration of S44819 at 0.1, 0.3 and 1 mg/kg, *p.o.* 120 min before the acquisition trial fully restored recognition memory in a dose-dependent manner (Fig. 3).

3.2 Remediation of ketamine-induced impairments of working and reference memory in the eight-arm radial maze in rats

Male rats were tested in the eight-arm maze in order to assess the effect of S44819 (0.3, 1 and 3 mg/kg, *p.o.*) in cognitive disruption induced by ketamine. Indeed, acute administration of ketamine (10 mg/kg) produced significant deterioration of working, as well as reference memory in the eight-arm radial maze (Fig. 4A & B). Acute administration of S44819 (Aqoat, 0.3 - 3 mg/kg *p.o.* 120 min before the trial) improved performance in both paradigms with 3 mg/kg being close to maximally effective.

3.3 Extinction of cued fear in mice

It has been reported that synaptic plasticity in the form of LTD, in addition to LTP are important for the extinction of cued fear (Myers and Davis, 2006; Nabavi et al., 2014). As S44819 facilitated hippocampal CA1 neuron LTP (Etherington et al., 2017) and as α5-$GABA_A$Rs may facilitate LTD (Martin et al., 2010), it was of interest to test whether or not S44819 had any effect on the extinction of cued fear by causing an imbalance of neuronal plasticity favouring LTP. The effect of S44819 on the extinction of cued fear was tested at 3 mg/kg daily*, i.p.* in mice (Fig. 5). Fear extinction was observed in vehicle as well as the S44819 treated groups. While in the control group the decrement in freezing behavior was significant only on day 5, in the S44819 treated groups there was a significant reduction on days 4 and 5. Taken together, S44819 (3mg/kg daily) had no deleterious effects on the extinction process, if anything, a small degree of enhancement of fear extinction was apparent on day 4.

3.4 Fear potentiated startle in rats

The potentiation of acoustic startle reflex by experimentally induced fear has been used as a preclinical surrogate of general anxiety disorder (Davis et al., 1993). As recent reports have suggested that α5-GABARs could mediate the anxiolytic effects of endogenous GABA (Behlke et al., 2016; Botta et al., 2015), an antagonist of these receptors, such as S44819 may prove to be

anxiogenic. Importantly, treatment with S44819 at 1 and 3 mg/kg produced a marked reduction of the enhancement of the startle response by the conditional light-pulse stimulus (Fig. 6), whereas no significant effect was observed at 10 mg/kg (Fig. 6). The compound (up to 10 mg/kg) had no effect of the basal startle reflex (data not shown). The reference anxiolytic compound diazepam (3mg/kg, i.p.) also significantly decreased the fear potentiated startle response of rats.

3.5 Social avoidance upon (electric shock) in rats

This test is a long-term model of social anxiety induced by a negative experience, which is sensitive to anxiolytic and anti-depressant drugs (Leveleki et al., 2006). A train of electric foot-shocks produced a significant level of social avoidance 24 h later (Fig. 7). Administration of S44819 (nanoF3, 0.3-1 mg/kg *i.p.*) 30 min before the social avoidance trial reversed the effect of prior electric shock (Fig. 7).

3.6 Social interaction in rats

The social interaction paradigm is used to evaluate the level of spontaneous anxiety in laboratory rodents. In male Wistar rats, the amount of time engaged in social interactions was dose-dependently enhanced by S44819 (1-10 mg/kp i.p.) (Fig. 8A). The lowest effective dose was 1 mg/kg *i.p.* (Fig. 8A). Importantly, no effect on exploration time was apparent for S44819 at doses up to 10 mg/kg (Fig. 8B). The effect of S44819 was equivalent to that produced by CDP (10 mg/kg *i.p.*), but note, that in contrast to S44819, CDP at this dose decreased the exploration time, *i.e*. had a sedative effect.

4. Discussion and conclusions

The data presented here demonstrate that S44819 can enhance normal learning and memory as well as ameliorate the impairment of these processes induced by acute, or sub-chronic inhibition of NMDA receptors by ketamine and PCP, respectively. Given the effects of S44819 to improve learning and memory, it was important to analyze its efficacy in tests of anxiety, fear and

depression. The results of the latter group of studies demonstrated that the anxiolytic efficacy of S44819 was manifested in paradigms that engaged a cognitive component in the behavior.

An extensive body of evidence has accumulated showing that a reduction of α5-$GABA_A$R function by pharmacological or genetic methods, improves cognitive performance in a variety of paradigms (Atack, 2011; Rudolph and Möhler, 2014). The pharmacons previously studied in this respect were all benzodiazepine site NAMs (Atack, 2011; Ballard et al., 2009; Chambers et al., 2003). In principle, S44819, a novel competitive blocker of α5-$GABA_A$Rs (Etherington et al., 2017) offers a potentially important advantage over α5-$GABA_A$R NAMs. Due to the relatively low affinity for its target, S44819 is likely to block the activation of extrasynaptic α5-$GABA_A$Rs exposed to maximally 1 µM of GABA, but to be largely ineffective against the high concentrations of GABA that occur in the synaptic cleft (Etherington et al., 2017; Ling et al., 2015). In contrast, α5-$GABA_A$R NAMs reduce the activity of synaptic (Salesse et al., 2011) as well as extrasynaptic α5-$GABA_A$Rs (Caraiscos et al., 2004; Glykys et al., 2008; Martin et al., 2010). Importantly, as expected of an α5-$GABA_A$R selective $GABA_A$ antagonist (Dawson et al., 2006) and in contrast to non-selective inhibitors of $GABA_A$ receptors, S44819 had no sedative or pro-convulsive effects.

The current study demonstrated the improvement of cognitive performance by S44819 in a variety of experimental paradigms. Long-term object recognition memory could be enhanced by S44819 in intact mice (present study) as well as rats (Etherington et al., 2017). Furthermore, similar to the effect of an α5-$GABA_A$ NAM in rats (Redrobe et al., 2012), the dramatic impairment of object recognition memory induced by subchronic PCP treatment of mice was reversed by acute treatment with S44819. Acute treatment with S44819 also improved declarative and working spatial memory disrupted by ketamine. Previously, it was also found to be effective against impairments of working memory induced by scopolamine (Etherington et al., 2017). Collectively, these results militate for actions of S44819 in the hippocampal gyrus and the neocortex. Indeed, previous work has shown that S44819 reduces tonic inhibition and potentiates LTP induced by a

theta-burst stimulation protocol in mouse CA1 pyramidal neurons (Etherington et al., 2017) and enhances the excitability of neurons in the M1 region of the human motor cortex (Darmani et al., 2016).

The outcomes of preclinical assays of cognitive performance may be markedly influenced by changes in the levels of learned and innate fear, or anxiety. Thus, we have examined the effect of S44819 in standard assays of anxiety and depression (see Suppl. Fig.-s 3-9). These studies revealed that S44819 was essentially without effect in these assays, except for the light-dark box paradigm where it had a significant anxiolytic effect at 30 mg/kg (Suppl. Fig. 5). In less standard assays, such as fear-potentiated startle, social interaction, and social avoidance induced by traumatic stress, the compound proved to be effective. Both fear-potentiated startle (Davis et al., 1993) and social interaction (File and Seth, 2003) are sensitive to the acute administration of anxiolytics such as diazepam and buspirone. However, in considering the interaction with the $GABA_AR$, S44819 is distinct from 1,4-benzodiazepines, being a competitive antagonist of the GABA recognition site (Etherington et al., 2017). Moreover, it shows no appreciable effect on any of the receptors known to interact with buspirone. Classic antidepressant compounds such as fluoxetine are anxiogenic when given acutely in the fear-potentiated startle, as well as the social interaction paradigms (Davis et al., 1993; File and Seth, 2003). It is clear that social-interaction involves early learning and memory experience that is recalled during the test (File and Seth, 2003), and may be facilitated by S44819. Fear-potentiated startle has an element of conditioned fear and anatomically involves the central amygdala (Davis et al., 1993; Walker and Davis, 1997). The facilitation by S44819 of cognitive surveillance of the conditioned stimulus-association could contribute to the reduction of startle amplitude. Furthermore, recent work has highlighted the involvement of tonic, α5-$GABA_AR$ mediated inhibition in regulating the activity of PKCδ expressing neurons in the central amygdala (Botta et al., 2015), which is a key brain site for fear conditioning (Ciocchi et al., 2010). Thus it cannot be excluded, that in addition to effects on cognitive

performance, neurons of the central amygdala are also substrates of the action of S44819 in the fear-potentiated startle paradigm.

Social avoidance produced by the single traumatic stress model described here, is diminished by antidepressants such as fluoxetine (Leveleki et al., 2006; Sziray et al., 2007). A rapid anti-depressant-like action of α5-GABA$_A$ NAMs to reduce social avoidance in animals subjected to chronic stress paradigms has been recently reported (Fischell et al., 2015). The efficacy of these compounds has been ascribed to the restoration of reduced AMPA receptor mediated synaptic transmission in the temporoammonic-hippocampal CA1 pathway (Fischell et al., 2015). Similar to other α5-GABA$_A$R inhibitors, S44819 enhanced theta-burst long-term potentiation in the CA1 area (Etherington et al., 2017). Thus, the parsimonious explanation for the efficacy of S44819 in social avoidance induced by traumatic stress is the potential for a dual mechanism of action at the level of associative memory as well as the affective filters in the amygdala resulting in the improvement of the behavioural response to stress. However, recent data (Botta et al., 2015) indicate, that by altering the excitability of neurons expressing α5-GABA$_A$R in the central amygdala, S44819 could also directly influence affective components of behaviour warranting further studies in this area.

In summary, we have demonstrated the enhancement as well as remediation of learning and memory by S44819 in several cognitive domains. The compound did not produce the side effects such as sedation, muscle weakness or pro-convulsive symptoms, which are characteristic of non-selective enhancers of GABA action. We also found that the S44819 has efficacy in preclinical assays of anxiety and traumatic stress that all have mnemonic components, *i.e*. the anxiolytic/anti-depressant-like efficacy of S44819 could be secondary to the facilitation of cognitive processes. Notably, S44819 has been shown to alter the EEG response to transcranial magnetic stimulation in humans (Darmani et al., 2016) and is now in Phase 2 clinical trials (https://www.clinicaltrialsregister.eu/ctr-search/trial/2016-001005-16/HU).

Acknowledgements We thank Dr Caroline Chemin, (Technologie Servier, Orleans, France) for the provision of formulated S44819, Dr Patricia Machado and Dr Elisabeth Mocaër (IDR Servier, Surennes, France) for critical comments on the manuscript.

Declaration of interests With the exception of JH, JJL and MS, all authors were employees of Egis Pharmaceuticals, PLC, Budapest, Hungary. MS was an employee of Les Laboratoires Servier, Surennes, Paris, France.

5. References

Atack, J. R., 2011. $GABA_A$ receptor subtype-selective modulators. II. alpha5-selective inverse agonists for cognition enhancement. Curr. Top. Med. Chem. 11, 1203-1214.

Ballard, T. M., Knoflach, F., Prinssen, E., Borroni, E., Vivian, J. A., Basile, J., Gasser, R., Moreau, J. L., Wettstein, J. G., Buettelmann, B., Knust, H., Thomas, A. W., Trube, G., Hernandez, M. C., 2009. RO4938581, a novel cognitive enhancer acting at $GABA_A$ alpha5 subunit-containing receptors. Psychopharmacology (Berl.) 202, 207-223.

Behlke, L. M., Foster, R. A., Liu, J., Benke, D., Benham, R. S., Nathanson, A. J., Yee, B. K., Zeilhofer, H. U., Engin, E., Rudolph, U., 2016. A Pharmacogenetic 'Restriction-of-Function' Approach Reveals Evidence for Anxiolytic-Like Actions Mediated by alpha5-Containing $GABA_A$ Receptors in Mice. Neuropsychopharmacology 41, 2492-2501.

Botta, P., Demmou, L., Kasugai, Y., Markovic, M., Xu, C., Fadok, J. P., Lu, T., Poe, M. M., Xu, L., Cook, J. M., Rudolph, U., Sah, P., Ferraguti, F., Luthi, A., 2015. Regulating anxiety with extrasynaptic inhibition. Nat. Neurosci. 18, 1493-1500.

Caraiscos, V. B., Elliott, E. M., You-Ten, K. E., Cheng, V. Y., Belelli, D., Newell, J. G., Jackson, M. F., Lambert, J. J., Rosahl, T. W., Wafford, K. A., MacDonald, J. F., Orser, B. A., 2004. Tonic inhibition in mouse hippocampal CA1 pyramidal neurons is mediated by alpha5 subunit-containing gamma-aminobutyric acid type A receptors. Proc. Natl. Acad. Sci. U. S. A. 101, 3662-3667.

Chambers, M. S., Atack, J. R., Broughton, H. B., Collinson, N., Cook, S., Dawson, G. R., Hobbs, S. C., Marshall, G., Maubach, K. A., Pillai, G. V., Reeve, A. J., MacLeod, A. M., 2003. Identification of a novel, selective GABA(A) alpha5 receptor inverse agonist which enhances cognition. J. Med. Chem. 46, 2227-2240.

Ciocchi, S., Herry, C., Grenier, F., Wolff, S. B., Letzkus, J. J., Vlachos, I., Ehrlich, I., Sprengel, R., Deisseroth, K., Stadler, M. B., Muller, C., Luthi, A., 2010. Encoding of conditioned fear in central amygdala inhibitory circuits. Nature 468, 277-282.

Darmani, G., Zipser, C. M., Bohmer, G. M., Deschet, K., Muller-Dahlhaus, F., Belardinelli, P., Schwab, M., Ziemann, U., 2016. Effects of the selective alpha5-$GABA_AR$ Antagonist S44819 on excitability in the human brain: A TMS-EMG and TMS-EEG Phase I study. J. Neurosci. 36, 12312-12320.

Davis, M., Falls, W. A., Campeau, S., Kim, M., 1993. Fear-potentiated startle: a neural and pharmacological analysis. Behav. Brain Res. 58, 175-198.

Dawson, G. R., Collinson, N., Atack, J. R., 2005. Development of subtype selective $GABA_A$ modulators. CNS Spectr 10, 21-27.

Dawson, G. R., Maubach, K. A., Collinson, N., Cobain, M., Everitt, B. J., MacLeod, A. M., Choudhury, H. I., McDonald, L. M., Pillai, G., Rycroft, W., Smith, A. J., Sternfeld, F., Tattersall, F. D., Wafford, K. A., Reynolds, D. S., Seabrook, G. R., Atack, J. R., 2006. An

inverse agonist selective for alpha5 subunit-containing $GABA_A$ receptors enhances cognition. J. Pharmacol. Exp. Ther. 316, 1335-1345.

Etherington, L. A., Pálvölgyi, A., Mihalik, B., Ling, I., Pallagi, K., Kertész, S., Gunn, B. G., Brown, A. R., Livesey, M. R., Belelli, D., Barkóczy, J., Varga, P., Spedding, M., Gacsályi, I., Antoni, F. A., Lambert, J. J., 2017. Selective targeting of extra-synaptic α5-$GABA_A$ receptors receptors by a new therapeutic agent S44819 (Egis-13529). Neuropharmacology submitted for publication.

File, S. E., Seth, P., 2003. A review of 25 years of the social interaction test. Eur. J. Pharmacol. 463, 35-53.

Fischell, J., Van Dyke, A. M., Kvarta, M. D., LeGates, T. A., Thompson, S. M., 2015. Rapid antidepressant action and restoration of excitatory synaptic strength after chronic stress by negative modulators of alpha5-containing $GABA_A$ receptors. Neuropsychopharmacology 40, 2499-2509.

Gacsályi, I., Nagy, K., Pallagi, K., Lévay, G., Hársing, L. G., Jr., Móricz, K., Kertész, S., Varga, P., Haller, J., Gigler, G., Szénási, G., Barkóczy, J., Biró, J., Spedding, M., Antoni, F. A., 2013. Egis-11150: a candidate antipsychotic compound with procognitive efficacy in rodents. Neuropharmacology 64, 254-263.

Glykys, J., Mann, E. O., Mody, I., 2008. Which GABA(A) receptor subunits are necessary for tonic inhibition in the hippocampus? J. Neurosci. 28, 1421-1426.

Grayson, B., Idris, N., Neill, J., 2007. Atypical antipsychotics attenuate a sub-chronic PCP-induced cognitive deficit in the novel object recognition task in the rat. Behav. Brain Res. 184, 31-38.

Klausberger, T., Somogyi, P., 2008. Neuronal diversity and temporal dynamics: the unity of hippocampal circuit operations. Science 321, 53-57.

Leveleki, C., Sziray, N., Levay, G., Barsvári, B., Soproni, K., Mikics, É., Haller, J., 2006. Pharmacological evaluation of the stress-induced social avoidance model of anxiety. Brain Res. Bull. 69, 153-160.

Ling, I., Barkóczy, J., Antoni, F., Gacsályi, I., Lévay, G., Spedding, M., Hársing, L., 2012. Dihydro-oxazolobenzodiazepinone compounds, a process for their preparation and pharmaceutical compositions containing them., US 2012/0232065 A1. Egis Pharmaceuticlas PLC, Les Laboratoires Servier, U.S.A.

Ling, I., Mihalik, B., Etherington, L. A., Kapus, G., Pálvölgyi, A., Gigler, G., Kertész, S., Gaál, A., Pallagi, K., Kiricsi, P., Szabó, E., Szénási, G., Papp, L., Hársing, L. G., Lévay, G., Spedding, M., Lambert, J. J., Belelli, D., Barkóczy, J., Volk, B., Simig, G., Gacsályi, I., Antoni, F. A., 2015. A novel GABA(A) alpha 5 receptor inhibitor with therapeutic potential. Eur. J. Pharmacol. 764, 497-507.

Martin, L. J., Zurek, A. A., MacDonald, J. F., Roder, J. C., Jackson, M. F., Orser, B. A., 2010. Alpha5GABA$_A$ receptor activity sets the threshold for long-term potentiation and constrains hippocampus-dependent memory. J. Neurosci. 30, 5269-5282.

Myers, K. M., Davis, M., 2006. Mechanisms of fear extinction. Mol. Psychiatry 12, 120-150.

Nabavi, S., Fox, R., Proulx, C. D., Lin, J. Y., Tsien, R. Y., Malinow, R., 2014. Engineering a memory with LTD and LTP. Nature 511, 348-352.

Pirker, S., Schwarzer, C., Wieselthaler, A., Sieghart, W., Sperk, G., 2000. GABA(A) receptors: immunocytochemical distribution of 13 subunits in the adult rat brain. Neuroscience 101, 815-850.

Redrobe, J. P., Bull, S., Plath, N., 2010. Translational aspects of the novel object recognition task in rats abstinent following sub-chronic treatment with phencyclidine (PCP):

Effects of modafinil and relevance to cognitive deficits in schizophrenia. Front Psychiatry 1, 146.

Redrobe, J. P., Elster, L., Frederiksen, K., Bundgaard, C., de Jong, I. E., Smith, G. P., Bruun, A. T., Larsen, P. H., Didriksen, M., 2012. Negative modulation of $GABA_A$ alpha5 receptors by RO4938581 attenuates discrete sub-chronic and early postnatal phencyclidine (PCP)-induced cognitive deficits in rats. Psychopharmacology (Berl.) 221, 451-468.

Rudolph, U., Möhler, H., 2014. $GABA_A$ receptor subtypes: Therapeutic potential in Down syndrome, affective disorders, schizophrenia, and autism. Annu. Rev. Pharmacol. Toxicol. 54, 483-507.

Salesse, C., Mueller, C. L., Chamberland, S., Topolnik, L., 2011. Age-dependent remodelling of inhibitory synapses onto hippocampal CA1 oriens-lacunosum moleculare interneurons. J. Physiol. 589, 4885-4901.

Stephens, D. N., Turski, L., 1993. Kindling to the benzodiazepine receptor inverse agonist, FG 7142: evidence for involvement of NMDA, but not non-NMDA, glutamatergic receptors. Neuropharmacology 32, 1011-1017.

Sziray, N., Leveleki, C., Lévay, G., Markó, B., Hársing, L. G., Jr., Mikics, É., Barsy, B., Haller, J., 2007. Mechanisms underlying the long-term behavioral effects of traumatic experience in rats: the role of serotonin/noradrenaline balance and NMDA receptors. Brain Res. Bull. 71, 376-385.

Ueno, S., Bracamontes, J., Zorumski, C., Weiss, D. S., Steinbach, J. H., 1997. Bicuculline and gabazine are allosteric inhibitors of channel opening of the $GABA_A$ receptor. J. Neurosci. 17, 625-634.

Walker, D. L., Davis, M., 1997. Double dissociation between the involvement of the bed nucleus of the stria terminalis and the central nucleus of the amygdala in startle

increases produced by conditioned versus unconditioned fear. J. Neurosci. 17, 9375-9383.

Walker, J. E., 1983. Glutamate, GABA, and CNS disease: a review. Neurochem. Res. 8, 521-550.

Wisden, W., Laurie, D. J., Monyer, H., Seeburg, P. H., 1992. The distribution of 13 $GABA_A$ receptor subunit mRNAs in the rat brain. I. Telencephalon, diencephalon, mesencephalon. J. Neurosci. 12, 1040-1062.

LEGENDS TO THE FIGURES

Fig. 1 A) *No appreciable effect of S44819 (nanoF3, up to at 30 mg/kg i.p) on spontaneous motor activity.* Data are mean ± S.E.M. n=8/group. B) *No apparent kindling effect of 40 mg/kg i.p. S44819 (nanoF3) in male NMRI mice after up to 19 days of treatment.* Note prominent epileptogenic kindling by pentylenetetrazole (see Supplement for methods). n=10/group.

Fig. 2 *Object recognition memory is enhanced by S44819 in intact NMRI mice.* The test trial with the new object was carried out 24 h after the acquisition trial with two identical objects. S44819 (Aqoat) was given p.o. 120 min before the acquisition trial. The discrimination index (DI) is shown where N is the time spent inspecting the new object, F is the time spent exploring the familiar object during the test trial. Data are mean ± S.E.M. n=10/group. ** P<0.01 one-way ANOVA followed by Dunnett's test *vs.* vehicle (0 mg/kg) group.

Fig. 3 *Reversal by S44819 of the impairment of object recognition memory induced by sub-chronic treatment with PCP.* NMRI mice were treated with saline or PCP 3 mg/kg twice daily *i.p.* for 2 x 5 days with a two-day pause between the two courses of treatment. Ten days after the last treatment with PCP object recognition memory was tested by examining novel object preference in the two-object version of the test. The test trial took place 15 min after the acquisition trial. Treatment with S44819 or vehicle (Aqoat, *p.o.)* was 120 min before the onset of acquisition. Data are mean ± S.E.M. n=10/group. ### P<0.001 Student's t-test vs Control (CTRL) group subchronically treated with saline and acutely receiving vehicle. ** P<0.01 ***P<0.001 one-way ANOVA followed by Dunnett's test vs vehicle (0 mg/kg) group with had received PCP.

Fig. 4 *The acute disruptive effect of 10 mg/kg ketamine on spatial reference (A) and working (B) memory is opposed by S44819.* A) Male Lister-Hooded rats were trained to criterion in the eight-arm radial maze for 13 days to retrieve bait placed in four of the arms. On the day of the test trial the animals received vehicle or S44819 (Aqoat, 0.3, 1, 3 mg/kg *p.o.*) 120 min prior to the start of the test. They were treated with vehicle or ketamine *i.p.* 30 min before the start of test. Data are median, n=9-10/group, the bars indicate the quartiles, ## P<0.01 *vs* control group receiving Aqoat vehicle and vehicle, Mann-Whitney U-test, ** P<0.01 Kruskal-Wallis ANOVA followed by Dunn's Multiple Comparison Test. B) Male Lister-Hooded rats were trained to criterion in the eight-arm radial maze for 18 days to retrieve bait placed in four of the arms. The location of the baits was changed before each trial. Drug treatments on the day of the trail were as for A. Data are median the bras indicate the quartiles, n=9-10/group. ## P<0.01 *vs.* control group receiving Aqoat vehicle and vehicle, Mann-Whitney U-test, ** P<0.01 Kruskal-Wallis ANOVA followed by Dunn's Multiple Comparison Test.

Fig. 5 *Effect of S44819 on the extinction of cued fear.* Male C57BL/6J mice were conditioned by pairing white noise with mild electric shock on day 1. The extinction of the freezing response to noise alone was followed over the next 4 days. Animals were injected with S44819 (nanoF3, 3 mg/kg *i.p.*) 30 min prior to each extinction session (indicated by upward arrows). Data were analysed by one-way ANOVA followed by Dunnett's test for multiple comparisons. ** P<0.01 *vs.* the respective values obtained on day 1.

Fig. 6 *Enhancement of social interaction in rats by S44819.* Pairs of male Wistar rats receiving the same pharmacological treatment were studied. Rats were pretreated with vehicle of S44819 *i.p.* 30 min prior to the start of the observation period with lasted 10 min. A) Time spent engaged in social interactions expressed as percentage of total observation time. B) Active exploration expressed as percentage of total observation time. ** P<0.01 when compared with the respective group receiving vehicle (0 mg/kg). Data were analyzed by Kruskal-Wallis ANOVA. The Mann-Whitney test was used for *post-hoc* comparisons. Sample size was 12, but one pair was discarded from the chlordiazepoxide group for technical reasons. Therefore, the sample size was 10 in this group.

Fig. 7 *Anxiolytic-like effect of S 4819 in the fear potentiated startle model in rats (male* Sprague-Dawley*) after acute i.p. administration.* Values are mean ± SEM (n=8-14 /group). # P <0.05 Student's two-tailed t-test between diazepam and the control group receiving vehicle to validate the test, * P<0.05 *vs.* group receiving vehicle (0 mg/kg), one-way ANOVA followed by Dunnett's *post-hoc* test for the doses of S44819 used.

Fig. 8 *Reversal of shock induced decrease in opponent time by S44819 in young male Wistar rats.* Treatment with vehicle of S44819 (nano F3) was given 30 min before the start of the social avoidance paradigm. Values are mean ± S.E.M. (n=8-9) ##: p<0.01, test validation, Student's t-test versus non-shocked control, * P<0.05 versus shocked control (0 mg/mkg). One-way ANOVA followed by Dunnett's *post-hoc* test for multiple comparisons.

Figure 1

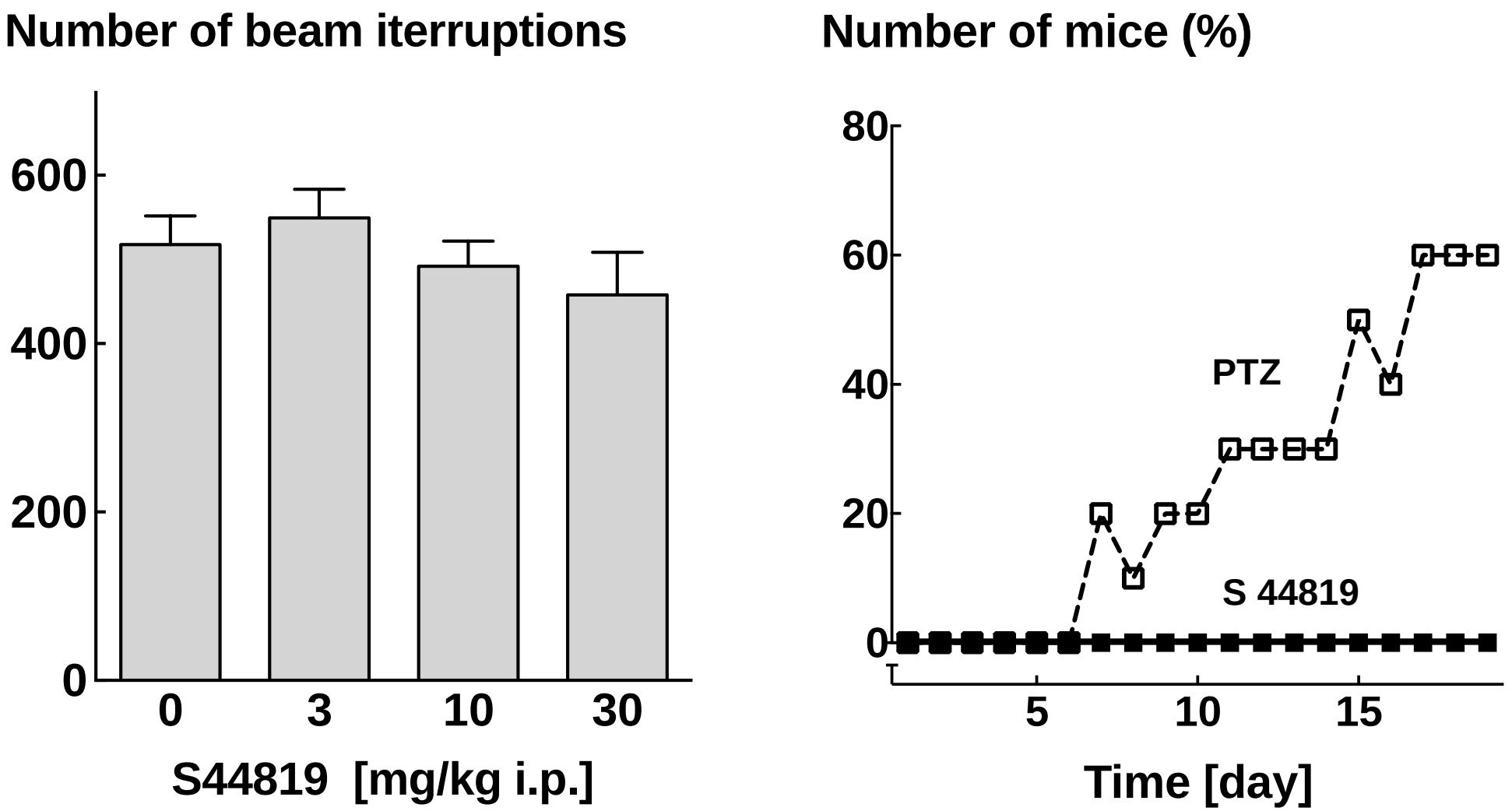

Number of beam iterruptions
600
400
200
0
0
3
10
30
S44819 [mg/kg i.p.]
Number of mice (%)
80
60
40
20
0
PTZ
S 44819
5
10
15
Time [day]

Figure 2

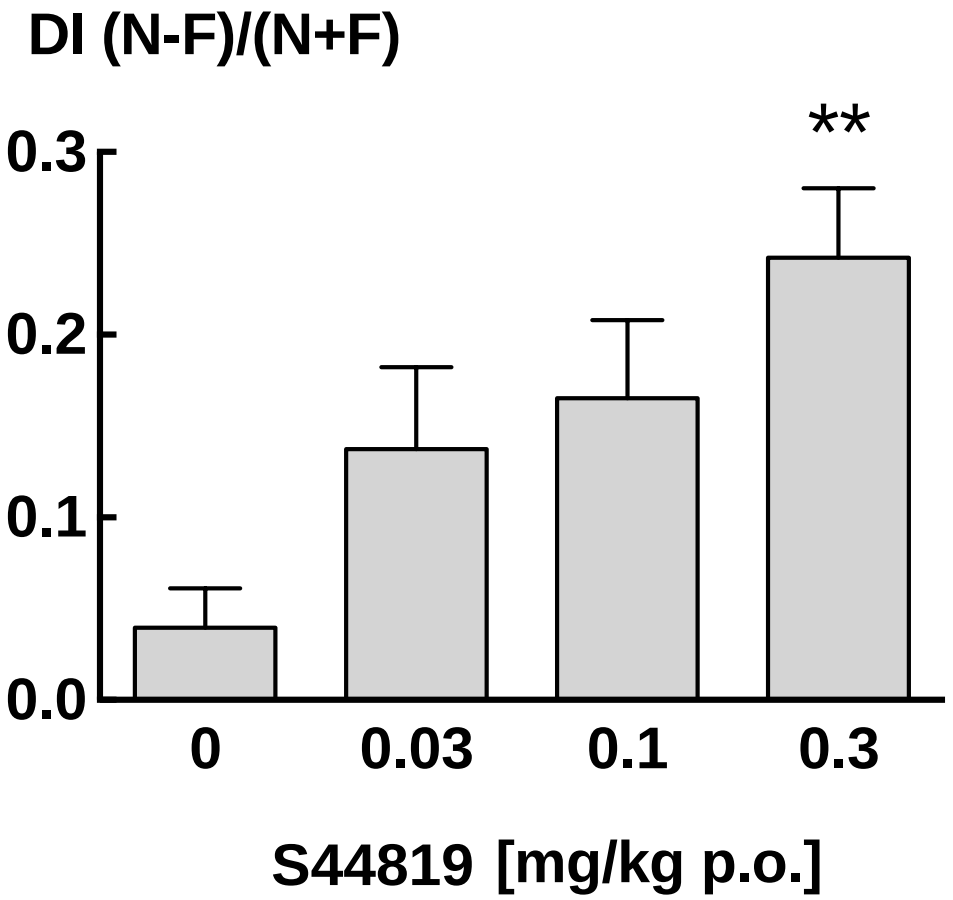


Figure 3

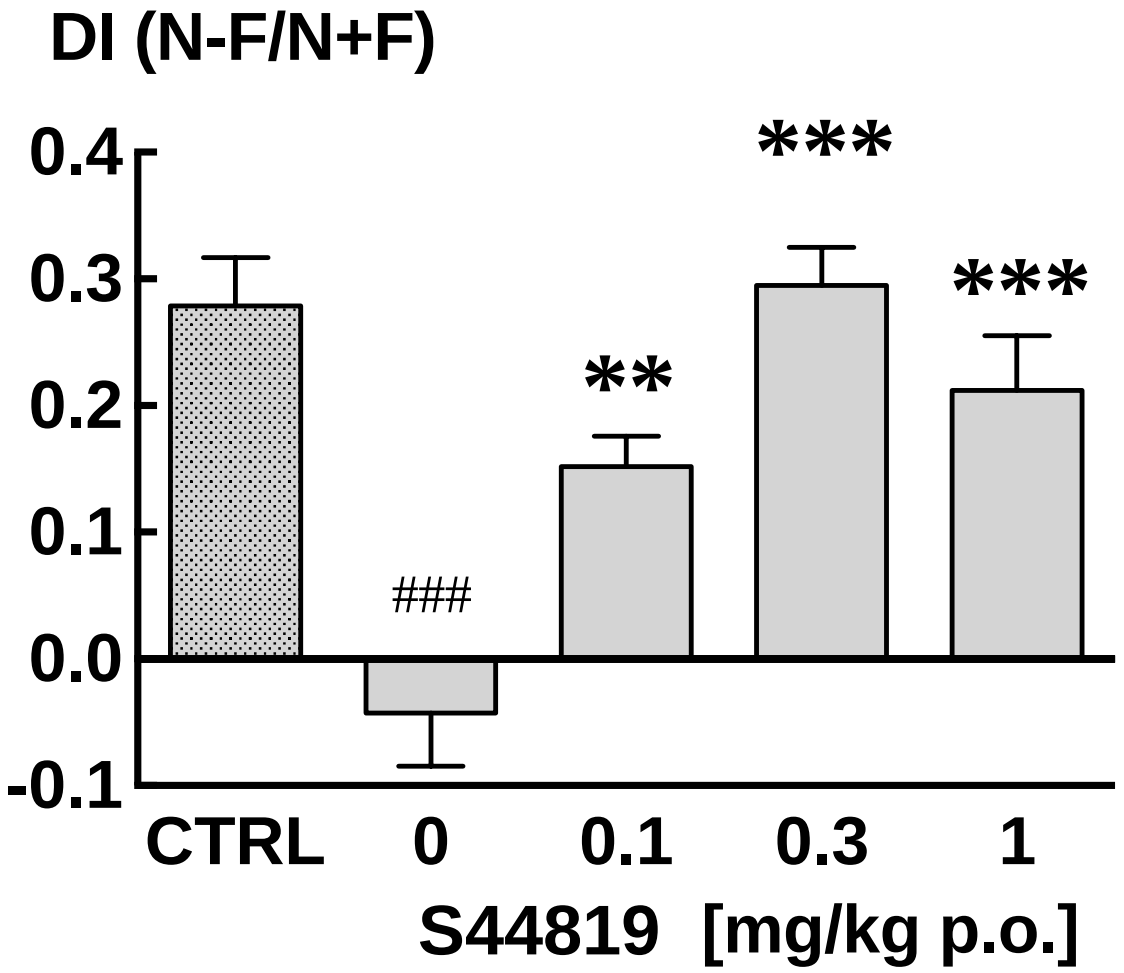


Figure 4

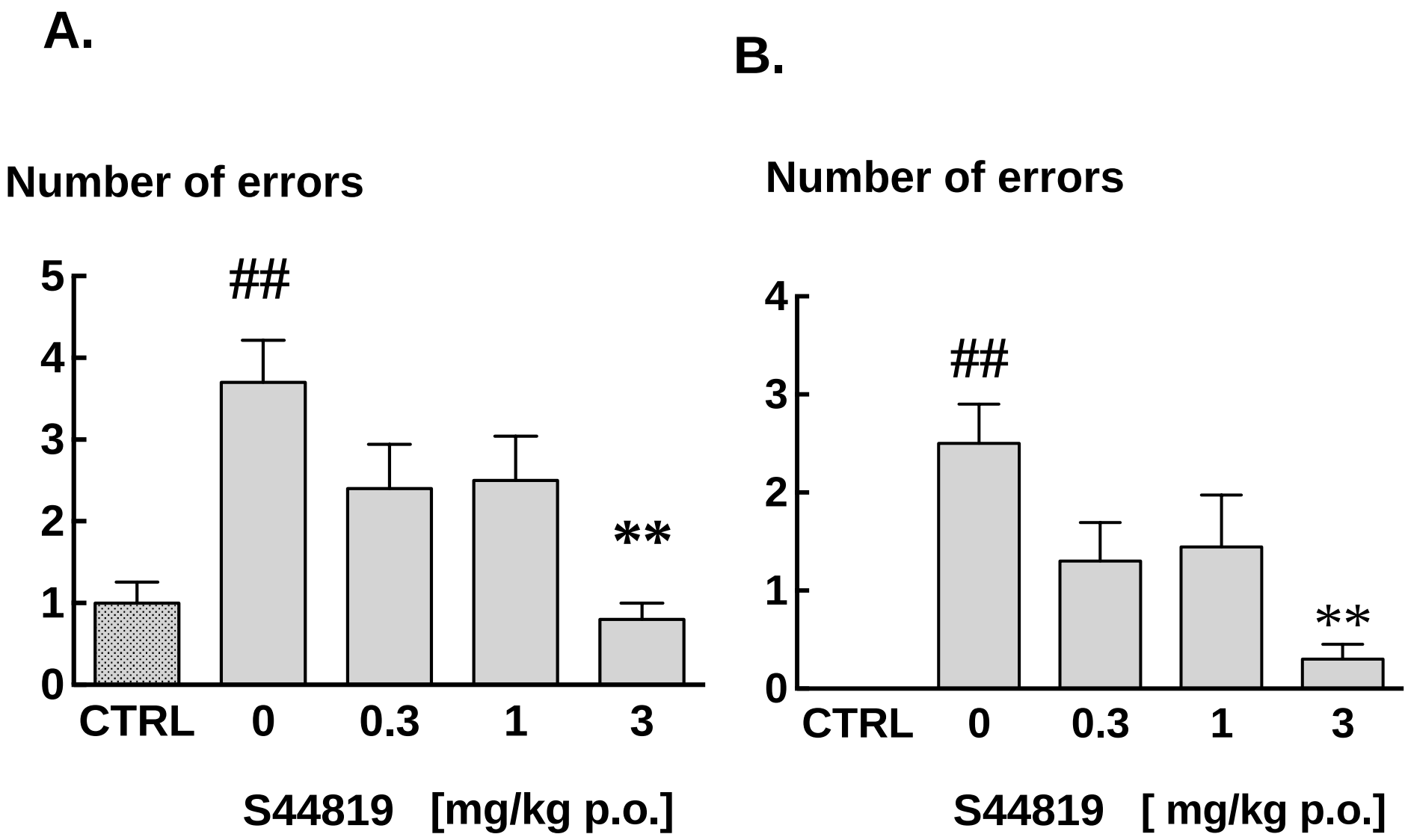

A.
Number of errors
5
4
3
2
1
0
##
**
CTRL
0
0.3
1
3
S44819 [mg/kg p.o.]
B.
Number of errors
4
3
2
1
0
##
**
CTRL
0
0.3
1
3
S44819 [mg/kg p.o.]

Figure 5

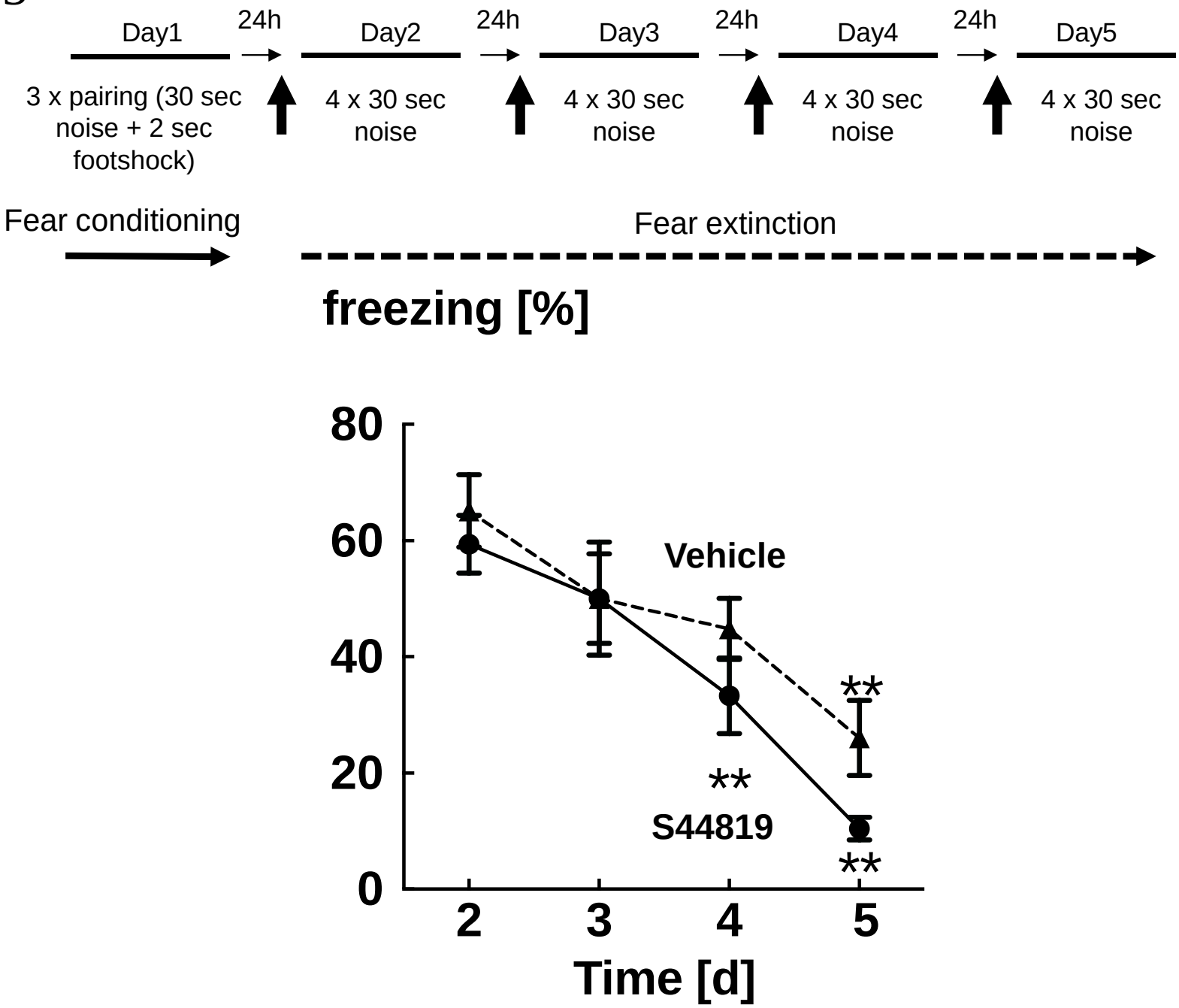

Day1
24h
Day2
24h
Day3
24h
Day4
24h
Day5
3 x pairing (30 sec noise + 2 sec footshock)
4 x 30 sec noise
4 x 30 sec noise
4 x 30 sec noise
4 x 30 sec noise
Fear conditioning
Fear extinction
freezing [%]
80
60
40
20
0
Vehicle
S44819
**
**
**
2
3
4
5
Time [d]

Figure 6

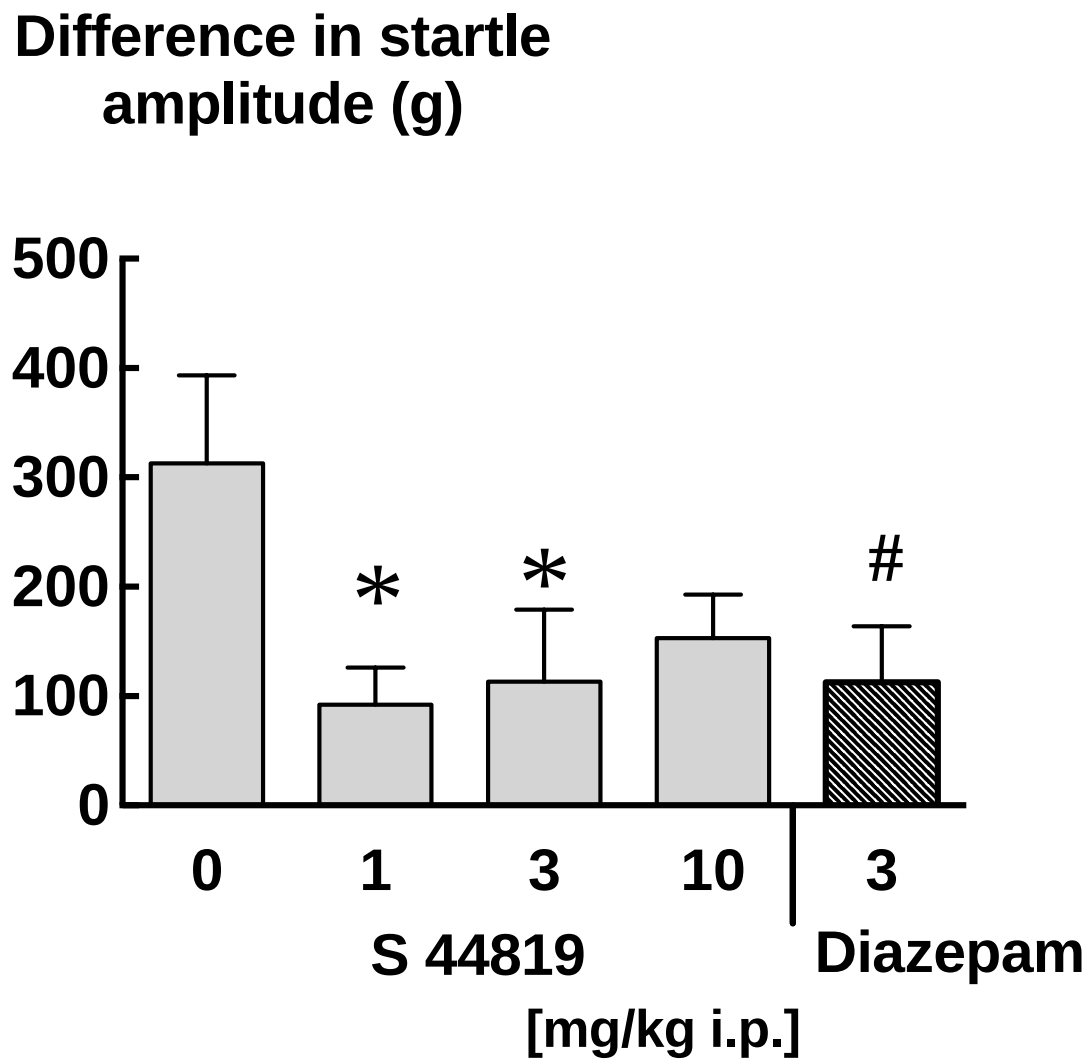

Difference in startle amplitude (g)
500
400
300
200
100
0
*
*
#
0
1
3
10
3
S 44819
Diazepam
[mg/kg i.p.]

Figure 7

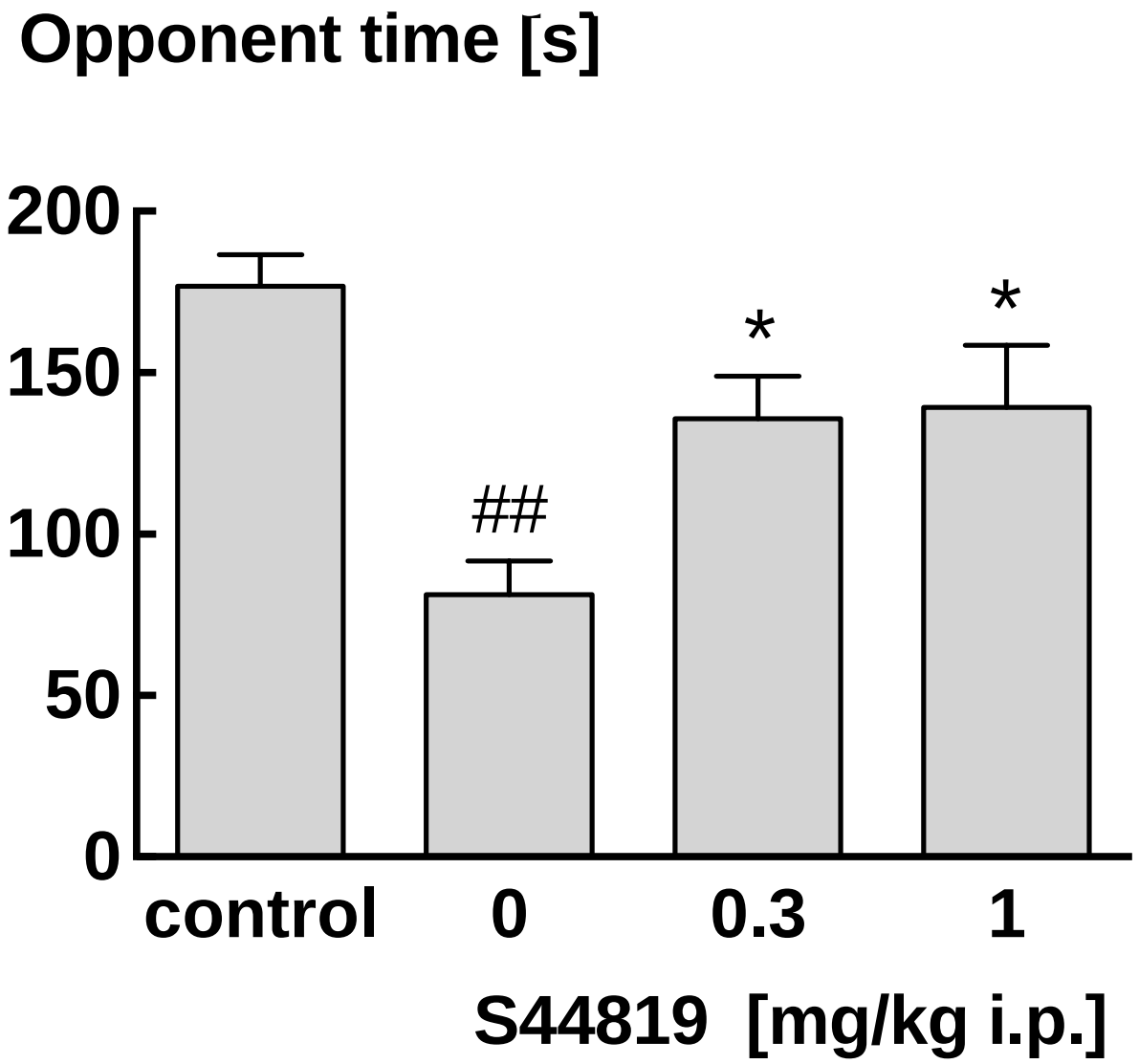
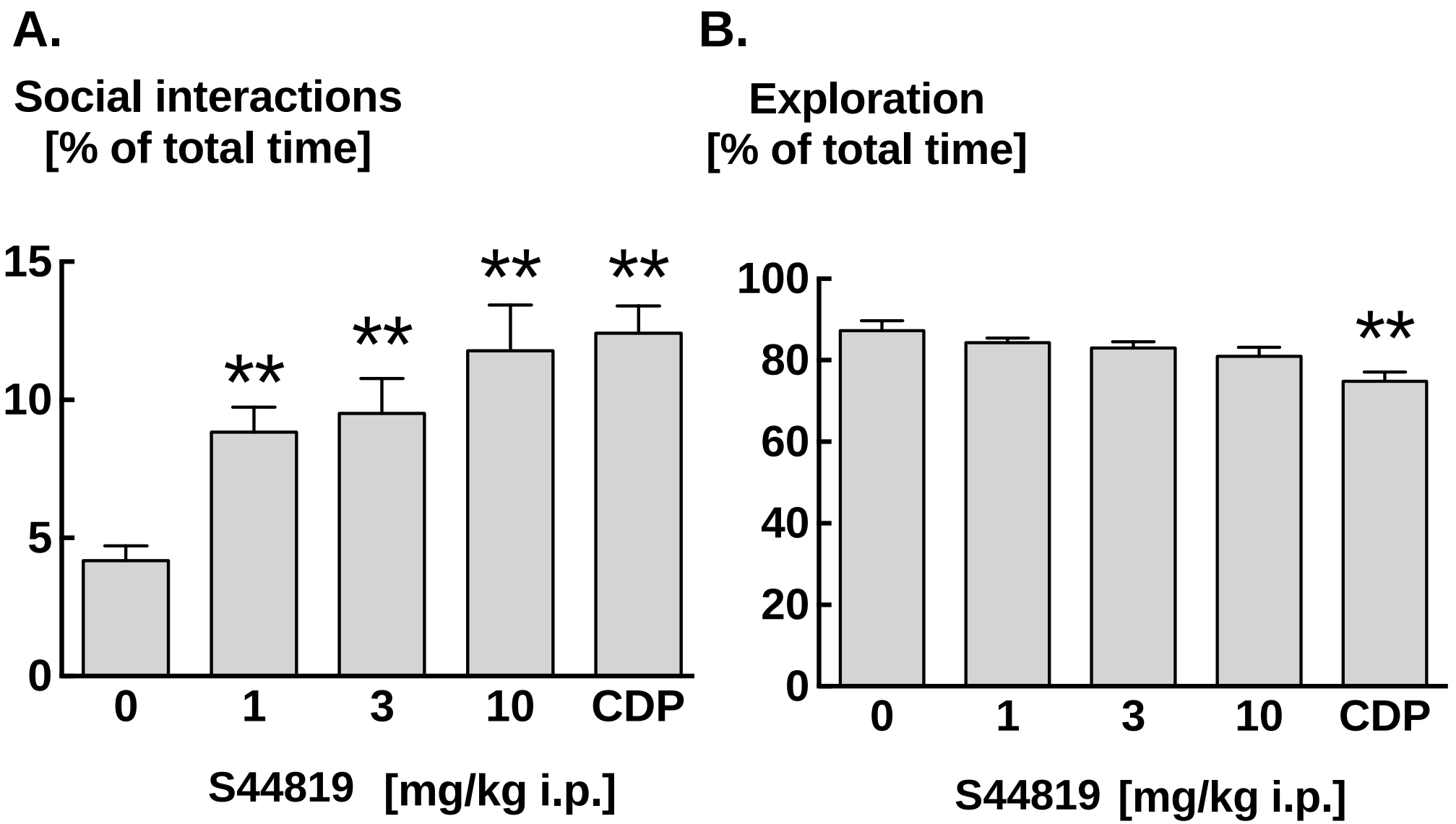


Figure 8


A.
Social interactions
[% of total time]
15
10
5
0
**
**
**
**
0
1
3
10
CDP
S44819 [mg/kg i.p.]
B.
Exploration
[% of total time]
100
80
60
40
20
0
**
0
1
3
10
CDP
S44819 [mg/kg i.p.]

Highlights:

• A novel competitive inhibitor selective for extrasynaptic α5-$GABA_A$ receptors (S44819) was tested in behavioral paradigms.

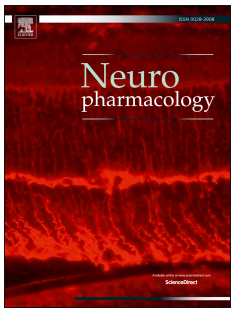
• The compound was effective in improving long-term as well as short-term (working) memory in mice and rats.

• It was effective in reversing the impairment of object recognition memory induced by sub-chronic treatment of mice with phencyclidine.

• S44819 also showed significant anxiolytic/antidepressant-like activity in tests that have a mnemonic component.

• The compound is now in Phase 2 clinical trials.

Accepted Manuscript



Behavioural pharmacology of the α5-GABA$_A$ receptor antagonist S44819: Enhancement and remediation of cognitive performance in preclinical models

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PII: S0028-3908(17)30312-X

DOI: 10.1016/j.neuropharm.2017.07.005

Reference: NP 6774

To appear in: *Neuropharmacology*

Received Date: 11 February 2017

Revised Date: 4 July 2017

Accepted Date: 6 July 2017

Please cite this article as: Gacsályi, Istvá., Móricz, K., Gigler, Gá., Wellmann, Já., Nagy, K., Ling, Istvá., Barkóczy, Jó., Haller, Jó., Lambert, J.J., Szénási, Gá., Spedding, M., Antoni, F.A., Behavioural pharmacology of the α5-GABA$_A$ receptor antagonist S44819: Enhancement and remediation of cognitive performance in preclinical models, *Neuropharmacology* (2017), doi: 10.1016/j.neuropharm.2017.07.005.

Behavioural pharmacology of the α5-GABA$_A$ receptor antagonist S44819: enhancement and remediation of cognitive performance in preclinical models.

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ABSTRACT

Previous work has shown that S44819 is a novel $GABA_A$ receptor ($GABA_AR$) antagonist, which is selective for extrasynaptic $GABA_ARs$ incorporating the α5 subunit (α5-$GABA_ARs$). The present study reports on the preclinical neuropsychopharmacological profile of S44819. Significantly, no sedative or pro-convulsive side effects of S44819 were found at doses up to 30 mg/kg *i.p.*. Object recognition (OR) memory in intact mice was enhanced by S44819 (0.3 mg/kg *p.o.*) given before the acquisition trial. Mice treated with phencyclidine for two weeks and tested six days after the cessation of treatment failed to show OR memory. This deficit was corrected by a single administration of S44819 (0.1, 0.3 or 1 mg/kg *p.o.*) prior to the acquisition trial. The amnestic effect of ketamine in rats tested in the eight-arm radial maze (reference and working memory versions) was blocked by S44819 (3 mg/kg *p.o.*). Extinction of cued fear was preserved during treatment with S44819 (3 mg/kg/*diem i.p.*). Administration of S44819 had no significant effect in the Vogel-conflict test, the elevated plus maze, the forced swim, the marble-burying and the tail-suspension tests. In contrast, anxiolytic/antidepressant-like effects of the compound were found in paradigms that have mnemonic components, such as social interaction, fear-potentiated startle and social avoidance induced by negative life experience. In summary, S44819 enhanced intact recognition memory and ameliorated memory deficits induced by inhibition of NMDA receptors. Anxiolytic/antidepressant efficacy was limited to paradigms involving cognitive function. In conclusion, S44819 is a novel psychoactive pro-cognitive compound with potential as a therapeutic agent in dementia.

1. Introduction

Gamma-aminobutyric acid (GABA) is a fundamentally important neurotransmitter in the CNS (Klausberger and Somogyi, 2008; Walker, 1983). The actions of GABA are mediated by ionotropic $GABA_A$ ($GABA_A$Rs) and metabotropic $GABA_B$ receptors. The topographical distribution of $GABA_A$R subunits in the brain indicates non-redundant functions (Pirker et al., 2000; Wisden et al., 1992). Indeed, in a pioneering studies analysing genetically engineered mice, the groups of Rudolph and Möhler and of McKernan and Whiting (reviewed by (Rudolph and Möhler, 2014)), showed that the diverse effects of diazepam on brain function were attributable to $GABA_A$Rs incorporating specific α-subunits. These studies were the first to show that α5-$GABA_A$Rs influence cognitive functions. Thus, the physiologic rationale of producing $GABA_A$ isoform selective pharmacons as therapeutic agents was established (Dawson et al., 2005).

The chemical synthesis and biological characterization of a family of tricyclic compounds, based on a 2,3-benzodiazepine scaffold, that are competitive inhibitors of GABA binding at the α–β subunit interface, was previously reported (Etherington et al., 2017; Ling et al., 2015). For some compounds, orthosteric antagonism was paired with inhibition of Cl^--channel gating, similar to the properties of the $GABA_A$R antagonist, bicuculline (Ueno et al., 1997). However, in contrast to bicuculline, which is a non-selective blocker of most $GABA_A$R isoforms, some of the novel compounds show considerable isoform selectivity. A potentially valuable compound in the series is S44819, which is a largely selective inhibitor of extrasynaptic α5-$GABA_A$Rs *in vitro* (Etherington et al., 2017). The current paper reports the *in vivo* preclinical pharmacology of this molecule. The results show enhancement of normal as well as remediation of impaired cognitive function, including object recognition memory, spatial working and reference memory by S44819. The compound also showed significant anxiolytic/antidepressant-like activity in tests that have a mnemonic component.

2. Methods

2.1 Animals

Male rats (190–270 g BW), or mice (20-30 g BW) — the strains are specified under the headings of the respective tests — were housed under standardized laboratory conditions (24 ± 2 ºC, 40–60% relative humidity), on a 12-h light/dark cycle with light onset at 6:00 AM. All experimental protocols were approved by the Animal Care and Use Ethical Committee of Egis Pharmaceuticals PLC and complied with the Hungarian Law of Animal Care and Use (1998. XVIII).

2.2 Compounds

S44189 (Egis-13529), substituted *8-methyl*-5-[*1-benzothiophen-2-yl*]-*1*,*9-dihydro-2H-[1*,*3*]*oxazolo* [*4*,*5-h][2*,*3*]*benzodiazepin-2-one* was produced as free base at greater than 97% purity at Egis Pharmaceuticals PLC as previously described (Ling et al., 2012; Ling et al., 2015). The solubility of the compound in physiological buffers containing 0.1% DMSO (v/v) was around 50 µM. Furthermore, because of the poor oral bioavailability of S44819 (F < 2%) the compound had to be micro-formulated for preclinical studies. Two formulations of S44819 were provided by Dr Caroline Chemin, Technologie Servier, (Orleans, France). The first, Aqoat, was a suspension of micro-milled S44819/Hypromellose Acetate Succinate (Shin Etsu Chemical Co, Japan)/Magnesium stearate (30/69.5/0.5, w/w/w) suspended in 2% (w/v) hydroxy-ethylcellulose (Molar Chemicals Ltd, Budapest). The second, (nanoF3) was a nano-encapsulated formulation consisting of S44819 3.85/Lipoïd S100 (Lipoïd GmbH, Ludwigshafen, Germany) 38.5/trehalose 57.7, w/w/w). Both formulations were used for intra-peritoneal (i.p.) as well as oral (p.o.) administration. The preparations were administered on the basis of their free base active product ingredient content. An effort was made to minimize the exposure of the animals to the excipients. Thus, the amount of excipient used depended on the highest dose of the drug administered in a given experiment — importantly, the dose of excipient was kept constant within a particular study. The highest dose of S44819 formulated with Aqoat was 30 mg/kg, thus all animals in received 0.50 mg/kg magnesium

stearate, 71 mg/kg hypromellose acetate succinate and 0.1 mg/kg of hydroxy-ethylcellulose. The dose of excipients was kept constant with reduced doses of drug. In the case of nanoF3, the highest dose used was 30 mg/kg of S44819, thus the animals also received 300 mg/kg Lipoïd S100 and 450mg/kg of trehalose. The dose of excipients was kept constant with reduced doses of drug. The drug administration regimes described below were optimized on the basis of preliminary kinetic studies in mice and rats with both formulations of S44819. Notably, both formulations gave similar brain:plasma concentration ratios approximating 1:2 in male mice (see Suppl. Fig.-s 1 and 2) and 1:4 in male rats (data not shown).

2.3 Object recognition assay in mice

Male albino NMRI mice bred in house were used. The experimental protocol was analogous to that described for rats (Gacsályi et al., 2013). Briefly, Day 0, familiarization: mice spent 2.5 minutes in a 24 x 34 x 24 cm box with black plastic walls. Acquisition: on the next day mice were allowed to explore freely two identical objects placed symmetrically at 4-4 cm from the shorter sides of the box. The animals were removed from the box when exploration times of both objects reached 10 s/object during a maximum 5 min period. Retention: 24 h later the animals were allowed to explore a novel object and an identical copy of the familiar object — the exploration time for both objects was recorded for 4 min. Treatment with Aqoat-formulated S44819 (0.03, 0.1, 0.3 mg/kg), or vehicle was performed *p.o.* 120 min before the start of the acquisition trial. Data were analysed by one-way analysis of variance followed by Dunnett's multiple comparison test.

In another series of experiments NMRI mice were subjected to sub-chronic treatment with vehicle (0.9% w/v NaCl), or phencyclidine (PCP, supplied by Tocris, U.K.): 3 mg/kg *i.p.* twice a day for a total of ten days (2x 5 days with a two-day break). Six days after the last PCP treatment, S44819 at 0.1, 0.3 and 1 mg/kg was administered *p.o.* 120 min before the start of the acquisition trial. The study protocol was exactly the same as above, except that the retention trial took place 15 min after the start of the acquisition period. Animals were returned to their home cage between the acquisition and retention trials. Data were analysed by Student’s t-test to validate the effect of PCP

(control vs. vehicle i.e. 0 mg/kg S44819-group) and one-way ANOVA followed by Dunnett's multiple comparison test (vehicle *vs.* S44819-treated groups) to assess the effect of S44819.

2.4 Working memory in the eight-arm radial maze in rats

Male Lister-Hooded rats 185-228 g body weight (Harlan Laboratories, The Netherlands) were trained and tested in an eight-arm-maze-based spatial working memory task in a dimly lit special room where geometric shapes were placed on the walls as visual cues. (Gacsályi et al., 2013). The maze was constructed from Perspex. It consisted of a central octagonal platform (diameter: 28 cm) and 8 radial direction arms (width: 9.5 cm, length: 70 cm, wall height: 12 cm). The central part and the arms had removable Perspex covers. Bait-food (5 mm diameter pellets made from a mixture (1:1, w/w) of ground tea-biscuits (Győri Otthon Keksz, Tesco stores, Budapest) and instant cocoa powder (Nesquik, Nestlé, Tesco stores)) was placed in a cavity at end of the arms. It was verified in previous independent studies, that the animals used the visual cues to find the bait**.** Young adult male Lister-Hooded rats were handled daily by the experimenter and were relatively food-deprived receiving approximately 8-10 g standard rodent food/day/animal per day. Training continued for 13 days (suspended on weekends). Rats were placed onto the central platform of the maze with all 8 arms baited. Rats were allowed to eat the bait from all 8 arms. If the rat did not eat all of the baits within 20 min it was removed from the maze. The following parameters were recorded. Repetition error (RE) is visiting the same arm more than once, Initial correct responses (ICR) is number of correct entries until first error. Animals included in the study had RE values <3 or ICR ≥7. On the last day of the experiment (after 13 days training) animals were given vehicle, or Aqoat-formulated S44819 *p.o*. (0.3, 1 and 3 mg/kg) and ketamine 10 mg/kg *i.p.*, at 120 and 30 min before the start of the trial, respectively. A single trial lasted a maximum of 5 min. The numbers of RE-s were analyzed by non-parametric Mann-Whitney test (intact control vs. ketamine control group) and Kruskal-Wallis test, followed by Dunn's multiple comparison test (ketamine control group vs. ketamine + different S44819 doses)

2.5 Reference memory in the eight-arm radial maze in rats

Reference memory was studied in male Lister-Hooded rats 230-310 g body weight with the same equipment and protocol as working memory (described above) with the following modifications. Only four arms out of eight were baited in the training sessions. Baiting was arranged in ten different configurations to the eight arms, a given animal was trained on the same configuration of arms throughout the entire experiment. Rats were allowed to eat the bait from all 4 arms. If the rat did not consume all the baits within 20 min it was removed from the maze. Training runs were performed once a day for 18 days (excluding weekends). On the last day of the experiment (after 18 days training) the animals were given vehicle, or Aqoat-formulated S44819 (0.3, 1 and 3 mg/kg, *p.o.)* and ketamine 10 mg/kg *i.p.*, at 120 and 30 min before the start of the trial, respectively. The numbers of errors, i.e. the number of entries into arms that had never been baited and the number of the missed baited arms within the 5 min of testing, were recorded. Inclusion criteria: **1)** the rat performs perfectly at least once in the last 3 learning days within 5 min, or **2**) the rat has less than 3 errors, or makes a mistake after the 3 correct entries in the last learning day in 5 or less minutes. The numbers of errors were analyzed by the Mann-Whitney U-test and the Kruskal-Wallis ANOVA followed by Dunn's multiple comparison test.

2.6 Extinction of cued fear

The subjects were male young adult C57BL/6J mice (20-25 g BW, Charles River, Germany). Apparatus: TSE Fear Conditioning System (TSE System GmbH, Bad Homburg, Germany), four animals were tested simultaneously. The protocol is summarized in Fig.5. Fear conditioning (Day 1): Mice were placed into the test chambers (21.5 cm x 21.5 cm x 35 cm) and after 3 min habituation were given the first of 3 noise/foot-shock pairings. The 0.7 mA, 2 s electric shock was delivered during the last 2 s of the 30 s exposure to white noise (85 dB). The average inter-trial interval was 2 min. Extinction procedure (Days 2 - 5): Twenty four h after the last trial, S44819 (3 mg/kg, nanoF3 formulated), or vehicle was administered *i.p*. each day 30 min before the start of

the extinction session. Mice were then placed into the same chamber where they received the conditioning and after 3 min habituation, were exposed to 4 x 30 s of white noise (85 dB) – inter-trial intervals were 60 s. The number of freezing reactions was recorded. The reaction was considered freezing if the mouse was immobile for a minimum 2 s. Freezing was scored by dividing the 30 s noise stimulus into 5 s bins. If the animal froze in all 6 bins it received a score of 6. Four noise stimuli were given in total and the percentage of bins in which freezing was evident was calculated for each animal. Data were analyzed by one-way ANOVA followed by Dunnett's test for multiple comparisons.

2.7 Fear potentiated startle response test in rats

Male Sprague-Dawley rats bred in house were used. The fear potentiated startle procedure (Davis et al., 1993; Walker and Davis, 1997) also see Suppl. Fig. 11, consisted of learning, pre-test and test periods. In the learning period over two consecutive days, male Sprague-Dawley rats were placed into the test chambers and 5 min later given the first of 10 light/foot-shock pairings. The 1 mA, 0.5 s shock was delivered during the last 0.5 s of the 3.7 s light pulse. The average inter-trial interval (ITI) was 3 min. Background noise: 55 dB. Twenty-four h after the learning period, rats were returned to the test chambers for the pre-test phase. Five min later 10 x 95 dB noise bursts (ITI: 20-40 s) were presented (habituation). After 30 s, 3 noise alone stimuli (95 dB) and 3 noise + light stimuli were presented. On the test day (24 h after the completion of pre-test) the rats were returned to the test chambers and 5 min later were presented with the same initial habituation noise. 30 s later 10-10 noise alone (95 dB) and noise + light stimuli in random order were presented to the animal. The calculated parameter is the difference in the magnitude of startle responses between "light + noise" and "noise alone" trials. (Difference = magnitude of startle response at "light-noise" trials – magnitude of startle response at "noise alone" trials). Acute treatment with vehicle, or S44819 (nanoF3, 1, 3 and 10 mg/kg *i.p.*), was on the test day 30 min before the trial. The positive control diazepam was administered acutely at 3 mg/kg, i.p. 30 min before the test. Data were analyzed by one-way ANOVA followed by Dunnett's test for multiple comparisons.

2.8 Social interaction in rats

The study was carried out on 60 male Wistar rats (Charles River Laboratories, Hungary) 190-210 g body weight. After their arrival in the animal facility, rats were housed in groups of four in cages measuring 45 x 30 x 20 cm. One week of habituation to local conditions was allowed. Rats were familiarized with the test apparatus by being placed individually into the test arena for 10 min on the last two days of the habituation. Rats were weighed on the last habituation day. Experiments were performed in two sessions on two consecutive days. Treatments were balanced over sessions. Pharmacological treatments were performed one day after the last individual exposure to the test arena, during the first half of the dark phase. The effects of vehicle and S44819 (nanoF3, 1 ,3 10 mg/kg *i.p.*) were compared together with 10 mg/kg chlordiazepoxide (CDP) as reference. Sample size was 12, but one pair was discarded from the chlordiazepoxide group for technical reasons. Therefore, the sample size was 10 in this group. Pairs of rats were placed in the test arena (60 x 50 x 40 cm) 30 min after drug treatment and observed for 10 min. The members of the pairs were unfamiliar to each other and received the same pharmacological treatment. Behavior was video-recorded through the transparent front wall of the test cage and later analyzed by means of a computer-based event recorder by an experimenter blind to the drug treatment regimen. The social interaction test arena was a dark grey plastic box of 40 x 60 x 50 cm with wood shaving bedding. Boxes were lit by white light. The front wall of the box was of transparent plastic. Six boxes were used in parallel. Fresh bedding was used for each pair of rats. The duration and the frequency of social interactions (sniffing partner; anogenital sniffing; following; allogrooming) and total amount time spent moving around the arena (exploration) were recorded. An increase in social interactions is consistent with an anxiolytic action. Increased resting and decreased exploration were considered signs of sedative or locomotor suppressive effects. The parameter analysed was the amount of time engaged in social interaction expressed

as a percentage of the total exploration time. Data were analyzed by Kruskal-Wallis ANOVA. The Mann-Whitney test was used for *post-hoc* comparisons.

2.9 Social avoidance induced by electric shock

The test was carried out in young male Wistar rats (7-9 weeks old, 250-350 g body weight) as previously reported (Leveleki et al., 2006; Sziray et al., 2007). Briefly, inescapable electric shocks (24 shocks, 3 mA, 1 s duration, 29 s interval) were delivered 1 day before the social avoidance test *via* the grid floor. Twenty-four h later, the social avoidance testing was performed in a different box from the one in which the foot shocks were delivered. Animals were injected *i.p.* with S44819 (nanoF3 0.3 and 1 mg/kg), or vehicle 30 min before the test. The subject was placed in a cage (16 x 52 cm) for a 3 min habituation period upon which a sliding door leading to a larger cage (40.5 x 41 cm) was opened. The larger cage was divided into two equal (40.5 cm x 20.5 cm) compartments by a transparent, perforated plastic wall running down the middle. The distant compartment contained a large, unfamiliar male Wistar rat (>11 weeks old, >400 g body weight), as the opponent. The opponent was un-shocked. The subject was allowed to explore the resulting space for 5 min. The test apparatus did not permit physical contact between the experimental and stimulus animals. The duration of visits made to the middle compartment (opponent time) was recorded and expressed as percentage of the total time of exploration (% opponent time). Data were analyzed by one-way ANOVA followed by Dunnett's test for multiple comparisons.

3. Results

Analysis of spontaneous locomotor activity (see Supplementary methods) indicated that nano-formulated S44819 had no sedative, or anxiogenic actions at doses up to 30 mg/kg *i.p.* (Fig. 1A). Further tests of anxiety were in agreement with this conclusion (Suppl. Figs. 4-7). Moreover, there were no signs of muscle weakness/relaxation in the inclined screen test at doses up to 100 mg/kg in marked contrast with Baclofen and Diazepam at 10ml/kg, *i.p.* both of which had a significant effect on muscle strength (Suppl. Fig. 3). Non-selective inhibition of $GABA_A$ receptors results in the lowering of the convulsion threshold (Stephens and Turski, 1993). This was indeed the case upon chronic administration of pentylenetetrazole (40 mg/kg *i.p,* see Supplementary methods): by day 18, 60 % of the animals displayed clonic seizures (Fig.1B). In contrast, there were no signs of seizures in the group given the same dosage of S44819 (Fig.1B).

3.1 Novel object preference in mice

In intact male mice, acute administration of Aqoat formulated S44819 at 0.3 mg/kg *p.o.,* given 120 min prior to the acquisition trial, significantly increased the time spent investigating the novel object over the familiar object 24 h after acquisition (Fig. 2). Note that in this study the time for object exploration and the inter-trial interval were adjusted so that the control animals had no significant retention of the object explored during the acquisition trial. Other dosage regimes of the drug were not tested.

The effect of S44819 on PCP-induced memory deficit was also examined. As reported previously by others (Grayson et al., 2007; Redrobe et al., 2010), mice subjected to sub-chronic treatment with PCP failed to show significant short-term object recognition memory (Fig. 3). Acute administration of S44819 at 0.1, 0.3 and 1 mg/kg, *p.o.* 120 min before the acquisition trial fully restored recognition memory in a dose-dependent manner (Fig. 3).

3.2 Remediation of ketamine-induced impairments of working and reference memory in the eight-arm radial maze in rats

Male rats were tested in the eight-arm maze in order to assess the effect of S44819 (0.3, 1 and 3 mg/kg, *p.o.*) in cognitive disruption induced by ketamine. Indeed, acute administration of ketamine (10 mg/kg) produced significant deterioration of working, as well as reference memory in the eight-arm radial maze (Fig. 4A & B). Acute administration of S44819 (Aqoat, 0.3 - 3 mg/kg *p.o.* 120 min before the trial) improved performance in both paradigms with 3 mg/kg being close to maximally effective.

3.3 Extinction of cued fear in mice

It has been reported that synaptic plasticity in the form of LTD, in addition to LTP are important for the extinction of cued fear (Myers and Davis, 2006; Nabavi et al., 2014). As S44819 facilitated hippocampal CA1 neuron LTP (Etherington et al., 2017) and as α5-$GABA_A$Rs may facilitate LTD (Martin et al., 2010), it was of interest to test whether or not S44819 had any effect on the extinction of cued fear by causing an imbalance of neuronal plasticity favouring LTP. The effect of S44819 on the extinction of cued fear was tested at 3 mg/kg daily*, i.p.* in mice (Fig. 5). Fear extinction was observed in vehicle as well as the S44819 treated groups. While in the control group the decrement in freezing behavior was significant only on day 5, in the S44819 treated groups there was a significant reduction on days 4 and 5. Taken together, S44819 (3mg/kg daily) had no deleterious effects on the extinction process, if anything, a small degree of enhancement of fear extinction was apparent on day 4.

3.4 Fear potentiated startle in rats

The potentiation of acoustic startle reflex by experimentally induced fear has been used as a preclinical surrogate of general anxiety disorder (Davis et al., 1993). As recent reports have suggested that α5-GABARs could mediate the anxiolytic effects of endogenous GABA (Behlke et al., 2016; Botta et al., 2015), an antagonist of these receptors, such as S44819 may prove to be

anxiogenic. Importantly, treatment with S44819 at 1 and 3 mg/kg produced a marked reduction of the enhancement of the startle response by the conditional light-pulse stimulus (Fig. 6), whereas no significant effect was observed at 10 mg/kg (Fig. 6). The compound (up to 10 mg/kg) had no effect of the basal startle reflex (data not shown). The reference anxiolytic compound diazepam (3mg/kg, i.p.) also significantly decreased the fear potentiated startle response of rats.

3.5 Social avoidance upon (electric shock) in rats

This test is a long-term model of social anxiety induced by a negative experience, which is sensitive to anxiolytic and anti-depressant drugs (Leveleki et al., 2006). A train of electric foot-shocks produced a significant level of social avoidance 24 h later (Fig. 7). Administration of S44819 (nanoF3, 0.3-1 mg/kg *i.p.*) 30 min before the social avoidance trial reversed the effect of prior electric shock (Fig. 7).

3.6 Social interaction in rats

The social interaction paradigm is used to evaluate the level of spontaneous anxiety in laboratory rodents. In male Wistar rats, the amount of time engaged in social interactions was dose-dependently enhanced by S44819 (1-10 mg/kp i.p.) (Fig. 8A). The lowest effective dose was 1 mg/kg *i.p.* (Fig. 8A). Importantly, no effect on exploration time was apparent for S44819 at doses up to 10 mg/kg (Fig. 8B). The effect of S44819 was equivalent to that produced by CDP (10 mg/kg *i.p.*), but note, that in contrast to S44819, CDP at this dose decreased the exploration time, *i.e*. had a sedative effect.

4. Discussion and conclusions

The data presented here demonstrate that S44819 can enhance normal learning and memory as well as ameliorate the impairment of these processes induced by acute, or sub-chronic inhibition of NMDA receptors by ketamine and PCP, respectively. Given the effects of S44819 to improve learning and memory, it was important to analyze its efficacy in tests of anxiety, fear and

depression. The results of the latter group of studies demonstrated that the anxiolytic efficacy of S44819 was manifested in paradigms that engaged a cognitive component in the behavior.

An extensive body of evidence has accumulated showing that a reduction of α5-$GABA_A$R function by pharmacological or genetic methods, improves cognitive performance in a variety of paradigms (Atack, 2011; Rudolph and Möhler, 2014). The pharmacons previously studied in this respect were all benzodiazepine site NAMs (Atack, 2011; Ballard et al., 2009; Chambers et al., 2003). In principle, S44819, a novel competitive blocker of α5-$GABA_A$Rs (Etherington et al., 2017) offers a potentially important advantage over α5-$GABA_A$R NAMs. Due to the relatively low affinity for its target, S44819 is likely to block the activation of extrasynaptic α5-$GABA_A$Rs exposed to maximally 1 µM of GABA, but to be largely ineffective against the high concentrations of GABA that occur in the synaptic cleft (Etherington et al., 2017; Ling et al., 2015). In contrast, α5-$GABA_A$R NAMs reduce the activity of synaptic (Salesse et al., 2011) as well as extrasynaptic α5-$GABA_A$Rs (Caraiscos et al., 2004; Glykys et al., 2008; Martin et al., 2010). Importantly, as expected of an α5-$GABA_A$R selective $GABA_A$ antagonist (Dawson et al., 2006) and in contrast to non-selective inhibitors of $GABA_A$ receptors, S44819 had no sedative or pro-convulsive effects.

The current study demonstrated the improvement of cognitive performance by S44819 in a variety of experimental paradigms. Long-term object recognition memory could be enhanced by S44819 in intact mice (present study) as well as rats (Etherington et al., 2017). Furthermore, similar to the effect of an α5-$GABA_A$ NAM in rats (Redrobe et al., 2012), the dramatic impairment of object recognition memory induced by subchronic PCP treatment of mice was reversed by acute treatment with S44819. Acute treatment with S44819 also improved declarative and working spatial memory disrupted by ketamine. Previously, it was also found to be effective against impairments of working memory induced by scopolamine (Etherington et al., 2017). Collectively, these results militate for actions of S44819 in the hippocampal gyrus and the neocortex. Indeed, previous work has shown that S44819 reduces tonic inhibition and potentiates LTP induced by a

theta-burst stimulation protocol in mouse CA1 pyramidal neurons (Etherington et al., 2017) and enhances the excitability of neurons in the M1 region of the human motor cortex (Darmani et al., 2016).

The outcomes of preclinical assays of cognitive performance may be markedly influenced by changes in the levels of learned and innate fear, or anxiety. Thus, we have examined the effect of S44819 in standard assays of anxiety and depression (see Suppl. Fig.-s 3-9). These studies revealed that S44819 was essentially without effect in these assays, except for the light-dark box paradigm where it had a significant anxiolytic effect at 30 mg/kg (Suppl. Fig. 5). In less standard assays, such as fear-potentiated startle, social interaction, and social avoidance induced by traumatic stress, the compound proved to be effective. Both fear-potentiated startle (Davis et al., 1993) and social interaction (File and Seth, 2003) are sensitive to the acute administration of anxiolytics such as diazepam and buspirone. However, in considering the interaction with the $GABA_AR$, S44819 is distinct from 1,4-benzodiazepines, being a competitive antagonist of the GABA recognition site (Etherington et al., 2017). Moreover, it shows no appreciable effect on any of the receptors known to interact with buspirone. Classic antidepressant compounds such as fluoxetine are anxiogenic when given acutely in the fear-potentiated startle, as well as the social interaction paradigms (Davis et al., 1993; File and Seth, 2003). It is clear that social-interaction involves early learning and memory experience that is recalled during the test (File and Seth, 2003), and may be facilitated by S44819. Fear-potentiated startle has an element of conditioned fear and anatomically involves the central amygdala (Davis et al., 1993; Walker and Davis, 1997). The facilitation by S44819 of cognitive surveillance of the conditioned stimulus-association could contribute to the reduction of startle amplitude. Furthermore, recent work has highlighted the involvement of tonic, α5-$GABA_AR$ mediated inhibition in regulating the activity of PKCδ expressing neurons in the central amygdala (Botta et al., 2015), which is a key brain site for fear conditioning (Ciocchi et al., 2010). Thus it cannot be excluded, that in addition to effects on cognitive

performance, neurons of the central amygdala are also substrates of the action of S44819 in the fear-potentiated startle paradigm.

Social avoidance produced by the single traumatic stress model described here, is diminished by antidepressants such as fluoxetine (Leveleki et al., 2006; Sziray et al., 2007). A rapid anti-depressant-like action of α5-GABA$_A$ NAMs to reduce social avoidance in animals subjected to chronic stress paradigms has been recently reported (Fischell et al., 2015). The efficacy of these compounds has been ascribed to the restoration of reduced AMPA receptor mediated synaptic transmission in the temporoammonic-hippocampal CA1 pathway (Fischell et al., 2015). Similar to other α5-GABA$_A$R inhibitors, S44819 enhanced theta-burst long-term potentiation in the CA1 area (Etherington et al., 2017). Thus, the parsimonious explanation for the efficacy of S44819 in social avoidance induced by traumatic stress is the potential for a dual mechanism of action at the level of associative memory as well as the affective filters in the amygdala resulting in the improvement of the behavioural response to stress. However, recent data (Botta et al., 2015) indicate, that by altering the excitability of neurons expressing α5-GABA$_A$R in the central amygdala, S44819 could also directly influence affective components of behaviour warranting further studies in this area.

In summary, we have demonstrated the enhancement as well as remediation of learning and memory by S44819 in several cognitive domains. The compound did not produce the side effects such as sedation, muscle weakness or pro-convulsive symptoms, which are characteristic of non-selective enhancers of GABA action. We also found that the S44819 has efficacy in preclinical assays of anxiety and traumatic stress that all have mnemonic components, *i.e*. the anxiolytic/anti-depressant-like efficacy of S44819 could be secondary to the facilitation of cognitive processes. Notably, S44819 has been shown to alter the EEG response to transcranial magnetic stimulation in humans (Darmani et al., 2016) and is now in Phase 2 clinical trials (https://www.clinicaltrialsregister.eu/ctr-search/trial/2016-001005-16/HU).

Acknowledgements We thank Dr Caroline Chemin, (Technologie Servier, Orleans, France) for the provision of formulated S44819, Dr Patricia Machado and Dr Elisabeth Mocaër (IDR Servier, Surennes, France) for critical comments on the manuscript.

Declaration of interests With the exception of JH, JJL and MS, all authors were employees of Egis Pharmaceuticals, PLC, Budapest, Hungary. MS was an employee of Les Laboratoires Servier, Surennes, Paris, France.

5. References

Atack, J. R., 2011. $GABA_A$ receptor subtype-selective modulators. II. alpha5-selective inverse agonists for cognition enhancement. Curr. Top. Med. Chem. 11, 1203-1214.

Ballard, T. M., Knoflach, F., Prinssen, E., Borroni, E., Vivian, J. A., Basile, J., Gasser, R., Moreau, J. L., Wettstein, J. G., Buettelmann, B., Knust, H., Thomas, A. W., Trube, G., Hernandez, M. C., 2009. RO4938581, a novel cognitive enhancer acting at $GABA_A$ alpha5 subunit-containing receptors. Psychopharmacology (Berl.) 202, 207-223.

Behlke, L. M., Foster, R. A., Liu, J., Benke, D., Benham, R. S., Nathanson, A. J., Yee, B. K., Zeilhofer, H. U., Engin, E., Rudolph, U., 2016. A Pharmacogenetic 'Restriction-of-Function' Approach Reveals Evidence for Anxiolytic-Like Actions Mediated by alpha5-Containing $GABA_A$ Receptors in Mice. Neuropsychopharmacology 41, 2492-2501.

Botta, P., Demmou, L., Kasugai, Y., Markovic, M., Xu, C., Fadok, J. P., Lu, T., Poe, M. M., Xu, L., Cook, J. M., Rudolph, U., Sah, P., Ferraguti, F., Luthi, A., 2015. Regulating anxiety with extrasynaptic inhibition. Nat. Neurosci. 18, 1493-1500.

Caraiscos, V. B., Elliott, E. M., You-Ten, K. E., Cheng, V. Y., Belelli, D., Newell, J. G., Jackson, M. F., Lambert, J. J., Rosahl, T. W., Wafford, K. A., MacDonald, J. F., Orser, B. A., 2004. Tonic inhibition in mouse hippocampal CA1 pyramidal neurons is mediated by alpha5 subunit-containing gamma-aminobutyric acid type A receptors. Proc. Natl. Acad. Sci. U. S. A. 101, 3662-3667.

Chambers, M. S., Atack, J. R., Broughton, H. B., Collinson, N., Cook, S., Dawson, G. R., Hobbs, S. C., Marshall, G., Maubach, K. A., Pillai, G. V., Reeve, A. J., MacLeod, A. M., 2003. Identification of a novel, selective GABA(A) alpha5 receptor inverse agonist which enhances cognition. J. Med. Chem. 46, 2227-2240.

Ciocchi, S., Herry, C., Grenier, F., Wolff, S. B., Letzkus, J. J., Vlachos, I., Ehrlich, I., Sprengel, R., Deisseroth, K., Stadler, M. B., Muller, C., Luthi, A., 2010. Encoding of conditioned fear in central amygdala inhibitory circuits. Nature 468, 277-282.

Darmani, G., Zipser, C. M., Bohmer, G. M., Deschet, K., Muller-Dahlhaus, F., Belardinelli, P., Schwab, M., Ziemann, U., 2016. Effects of the selective alpha5-$GABA_AR$ Antagonist S44819 on excitability in the human brain: A TMS-EMG and TMS-EEG Phase I study. J. Neurosci. 36, 12312-12320.

Davis, M., Falls, W. A., Campeau, S., Kim, M., 1993. Fear-potentiated startle: a neural and pharmacological analysis. Behav. Brain Res. 58, 175-198.

Dawson, G. R., Collinson, N., Atack, J. R., 2005. Development of subtype selective $GABA_A$ modulators. CNS Spectr 10, 21-27.

Dawson, G. R., Maubach, K. A., Collinson, N., Cobain, M., Everitt, B. J., MacLeod, A. M., Choudhury, H. I., McDonald, L. M., Pillai, G., Rycroft, W., Smith, A. J., Sternfeld, F., Tattersall, F. D., Wafford, K. A., Reynolds, D. S., Seabrook, G. R., Atack, J. R., 2006. An

inverse agonist selective for alpha5 subunit-containing $GABA_A$ receptors enhances cognition. J. Pharmacol. Exp. Ther. 316, 1335-1345.

Etherington, L. A., Pálvölgyi, A., Mihalik, B., Ling, I., Pallagi, K., Kertész, S., Gunn, B. G., Brown, A. R., Livesey, M. R., Belelli, D., Barkóczy, J., Varga, P., Spedding, M., Gacsályi, I., Antoni, F. A., Lambert, J. J., 2017. Selective targeting of extra-synaptic α5-$GABA_A$ receptors receptors by a new therapeutic agent S44819 (Egis-13529). Neuropharmacology submitted for publication.

File, S. E., Seth, P., 2003. A review of 25 years of the social interaction test. Eur. J. Pharmacol. 463, 35-53.

Fischell, J., Van Dyke, A. M., Kvarta, M. D., LeGates, T. A., Thompson, S. M., 2015. Rapid antidepressant action and restoration of excitatory synaptic strength after chronic stress by negative modulators of alpha5-containing $GABA_A$ receptors. Neuropsychopharmacology 40, 2499-2509.

Gacsályi, I., Nagy, K., Pallagi, K., Lévay, G., Hársing, L. G., Jr., Móricz, K., Kertész, S., Varga, P., Haller, J., Gigler, G., Szénási, G., Barkóczy, J., Biró, J., Spedding, M., Antoni, F. A., 2013. Egis-11150: a candidate antipsychotic compound with procognitive efficacy in rodents. Neuropharmacology 64, 254-263.

Glykys, J., Mann, E. O., Mody, I., 2008. Which GABA(A) receptor subunits are necessary for tonic inhibition in the hippocampus? J. Neurosci. 28, 1421-1426.

Grayson, B., Idris, N., Neill, J., 2007. Atypical antipsychotics attenuate a sub-chronic PCP-induced cognitive deficit in the novel object recognition task in the rat. Behav. Brain Res. 184, 31-38.

Klausberger, T., Somogyi, P., 2008. Neuronal diversity and temporal dynamics: the unity of hippocampal circuit operations. Science 321, 53-57.

Leveleki, C., Sziray, N., Levay, G., Barsvári, B., Soproni, K., Mikics, É., Haller, J., 2006. Pharmacological evaluation of the stress-induced social avoidance model of anxiety. Brain Res. Bull. 69, 153-160.

Ling, I., Barkóczy, J., Antoni, F., Gacsályi, I., Lévay, G., Spedding, M., Hársing, L., 2012. Dihydro-oxazolobenzodiazepinone compounds, a process for their preparation and pharmaceutical compositions containing them., US 2012/0232065 A1. Egis Pharmaceuticlas PLC, Les Laboratoires Servier, U.S.A.

Ling, I., Mihalik, B., Etherington, L. A., Kapus, G., Pálvölgyi, A., Gigler, G., Kertész, S., Gaál, A., Pallagi, K., Kiricsi, P., Szabó, E., Szénási, G., Papp, L., Hársing, L. G., Lévay, G., Spedding, M., Lambert, J. J., Belelli, D., Barkóczy, J., Volk, B., Simig, G., Gacsályi, I., Antoni, F. A., 2015. A novel GABA(A) alpha 5 receptor inhibitor with therapeutic potential. Eur. J. Pharmacol. 764, 497-507.

Martin, L. J., Zurek, A. A., MacDonald, J. F., Roder, J. C., Jackson, M. F., Orser, B. A., 2010. Alpha5GABA$_A$ receptor activity sets the threshold for long-term potentiation and constrains hippocampus-dependent memory. J. Neurosci. 30, 5269-5282.

Myers, K. M., Davis, M., 2006. Mechanisms of fear extinction. Mol. Psychiatry 12, 120-150.

Nabavi, S., Fox, R., Proulx, C. D., Lin, J. Y., Tsien, R. Y., Malinow, R., 2014. Engineering a memory with LTD and LTP. Nature 511, 348-352.

Pirker, S., Schwarzer, C., Wieselthaler, A., Sieghart, W., Sperk, G., 2000. GABA(A) receptors: immunocytochemical distribution of 13 subunits in the adult rat brain. Neuroscience 101, 815-850.

Redrobe, J. P., Bull, S., Plath, N., 2010. Translational aspects of the novel object recognition task in rats abstinent following sub-chronic treatment with phencyclidine (PCP):

Effects of modafinil and relevance to cognitive deficits in schizophrenia. Front Psychiatry 1, 146.

Redrobe, J. P., Elster, L., Frederiksen, K., Bundgaard, C., de Jong, I. E., Smith, G. P., Bruun, A. T., Larsen, P. H., Didriksen, M., 2012. Negative modulation of $GABA_A$ alpha5 receptors by RO4938581 attenuates discrete sub-chronic and early postnatal phencyclidine (PCP)-induced cognitive deficits in rats. Psychopharmacology (Berl.) 221, 451-468.

Rudolph, U., Möhler, H., 2014. $GABA_A$ receptor subtypes: Therapeutic potential in Down syndrome, affective disorders, schizophrenia, and autism. Annu. Rev. Pharmacol. Toxicol. 54, 483-507.

Salesse, C., Mueller, C. L., Chamberland, S., Topolnik, L., 2011. Age-dependent remodelling of inhibitory synapses onto hippocampal CA1 oriens-lacunosum moleculare interneurons. J. Physiol. 589, 4885-4901.

Stephens, D. N., Turski, L., 1993. Kindling to the benzodiazepine receptor inverse agonist, FG 7142: evidence for involvement of NMDA, but not non-NMDA, glutamatergic receptors. Neuropharmacology 32, 1011-1017.

Sziray, N., Leveleki, C., Lévay, G., Markó, B., Hársing, L. G., Jr., Mikics, É., Barsy, B., Haller, J., 2007. Mechanisms underlying the long-term behavioral effects of traumatic experience in rats: the role of serotonin/noradrenaline balance and NMDA receptors. Brain Res. Bull. 71, 376-385.

Ueno, S., Bracamontes, J., Zorumski, C., Weiss, D. S., Steinbach, J. H., 1997. Bicuculline and gabazine are allosteric inhibitors of channel opening of the $GABA_A$ receptor. J. Neurosci. 17, 625-634.

Walker, D. L., Davis, M., 1997. Double dissociation between the involvement of the bed nucleus of the stria terminalis and the central nucleus of the amygdala in startle

increases produced by conditioned versus unconditioned fear. J. Neurosci. 17, 9375-9383.

Walker, J. E., 1983. Glutamate, GABA, and CNS disease: a review. Neurochem. Res. 8, 521-550.

Wisden, W., Laurie, D. J., Monyer, H., Seeburg, P. H., 1992. The distribution of 13 $GABA_A$ receptor subunit mRNAs in the rat brain. I. Telencephalon, diencephalon, mesencephalon. J. Neurosci. 12, 1040-1062.

LEGENDS TO THE FIGURES

Fig. 1 A) *No appreciable effect of S44819 (nanoF3, up to at 30 mg/kg i.p) on spontaneous motor activity.* Data are mean ± S.E.M. n=8/group. B) *No apparent kindling effect of 40 mg/kg i.p. S44819 (nanoF3) in male NMRI mice after up to 19 days of treatment.* Note prominent epileptogenic kindling by pentylenetetrazole (see Supplement for methods). n=10/group.

Fig. 2 *Object recognition memory is enhanced by S44819 in intact NMRI mice.* The test trial with the new object was carried out 24 h after the acquisition trial with two identical objects. S44819 (Aqoat) was given p.o. 120 min before the acquisition trial. The discrimination index (DI) is shown where N is the time spent inspecting the new object, F is the time spent exploring the familiar object during the test trial. Data are mean ± S.E.M. n=10/group. ** P<0.01 one-way ANOVA followed by Dunnett's test *vs.* vehicle (0 mg/kg) group.

Fig. 3 *Reversal by S44819 of the impairment of object recognition memory induced by sub-chronic treatment with PCP.* NMRI mice were treated with saline or PCP 3 mg/kg twice daily *i.p.* for 2 x 5 days with a two-day pause between the two courses of treatment. Ten days after the last treatment with PCP object recognition memory was tested by examining novel object preference in the two-object version of the test. The test trial took place 15 min after the acquisition trial. Treatment with S44819 or vehicle (Aqoat, *p.o.)* was 120 min before the onset of acquisition. Data are mean ± S.E.M. n=10/group. ### P<0.001 Student's t-test vs Control (CTRL) group subchronically treated with saline and acutely receiving vehicle. ** P<0.01 ***P<0.001 one-way ANOVA followed by Dunnett's test vs vehicle (0 mg/kg) group with had received PCP.

Fig. 4 *The acute disruptive effect of 10 mg/kg ketamine on spatial reference (A) and working (B) memory is opposed by S44819.* A) Male Lister-Hooded rats were trained to criterion in the eight-arm radial maze for 13 days to retrieve bait placed in four of the arms. On the day of the test trial the animals received vehicle or S44819 (Aqoat, 0.3, 1, 3 mg/kg *p.o.*) 120 min prior to the start of the test. They were treated with vehicle or ketamine *i.p.* 30 min before the start of test. Data are median, n=9-10/group, the bars indicate the quartiles, ## P<0.01 *vs* control group receiving Aqoat vehicle and vehicle, Mann-Whitney U-test, ** P<0.01 Kruskal-Wallis ANOVA followed by Dunn's Multiple Comparison Test. B) Male Lister-Hooded rats were trained to criterion in the eight-arm radial maze for 18 days to retrieve bait placed in four of the arms. The location of the baits was changed before each trial. Drug treatments on the day of the trail were as for A. Data are median the bras indicate the quartiles, n=9-10/group. ## P<0.01 *vs.* control group receiving Aqoat vehicle and vehicle, Mann-Whitney U-test, ** P<0.01 Kruskal-Wallis ANOVA followed by Dunn's Multiple Comparison Test.

Fig. 5 *Effect of S44819 on the extinction of cued fear.* Male C57BL/6J mice were conditioned by pairing white noise with mild electric shock on day 1. The extinction of the freezing response to noise alone was followed over the next 4 days. Animals were injected with S44819 (nanoF3, 3 mg/kg *i.p.*) 30 min prior to each extinction session (indicated by upward arrows). Data were analysed by one-way ANOVA followed by Dunnett's test for multiple comparisons. ** P<0.01 *vs.* the respective values obtained on day 1.

Fig. 6 *Enhancement of social interaction in rats by S44819.* Pairs of male Wistar rats receiving the same pharmacological treatment were studied. Rats were pretreated with vehicle of S44819 *i.p.* 30 min prior to the start of the observation period with lasted 10 min. A) Time spent engaged in social interactions expressed as percentage of total observation time. B) Active exploration expressed as percentage of total observation time. ** P<0.01 when compared with the respective group receiving vehicle (0 mg/kg). Data were analyzed by Kruskal-Wallis ANOVA. The Mann-Whitney test was used for *post-hoc* comparisons. Sample size was 12, but one pair was discarded from the chlordiazepoxide group for technical reasons. Therefore, the sample size was 10 in this group.

Fig. 7 *Anxiolytic-like effect of S 4819 in the fear potentiated startle model in rats (male* Sprague-Dawley*) after acute i.p. administration.* Values are mean ± SEM (n=8-14 /group). # P <0.05 Student's two-tailed t-test between diazepam and the control group receiving vehicle to validate the test, * P<0.05 *vs.* group receiving vehicle (0 mg/kg), one-way ANOVA followed by Dunnett's *post-hoc* test for the doses of S44819 used.

Fig. 8 *Reversal of shock induced decrease in opponent time by S44819 in young male Wistar rats.* Treatment with vehicle of S44819 (nano F3) was given 30 min before the start of the social avoidance paradigm. Values are mean ± S.E.M. (n=8-9) ##: p<0.01, test validation, Student's t-test versus non-shocked control, * P<0.05 versus shocked control (0 mg/mkg). One-way ANOVA followed by Dunnett's *post-hoc* test for multiple comparisons.

Figure 1

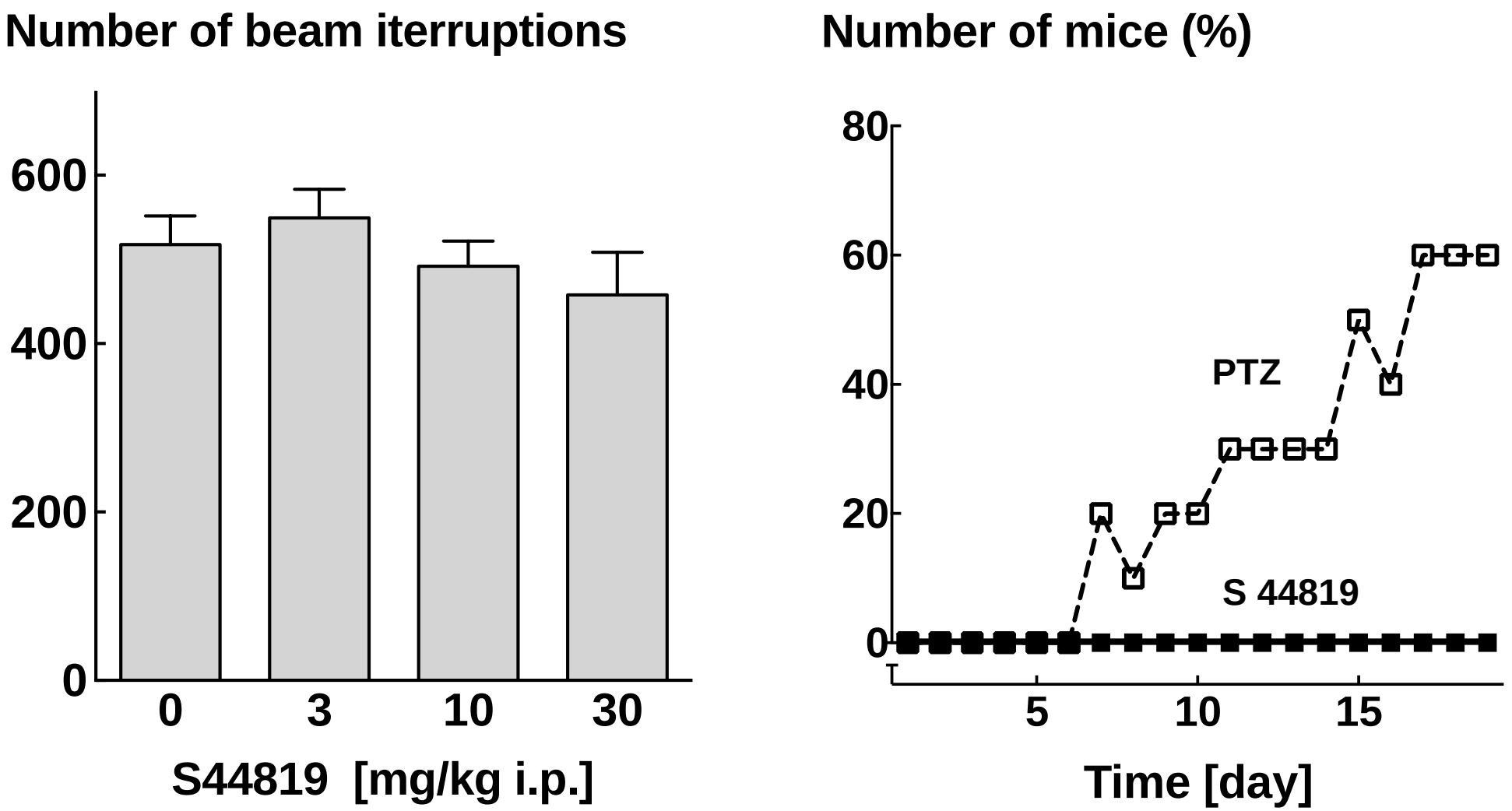

Number of beam iterruptions
600
400
200
0
0
3
10
30
S44819 [mg/kg i.p.]
Number of mice (%)
80
60
40
20
0
PTZ
S 44819
5
10
15
Time [day]

Figure 2

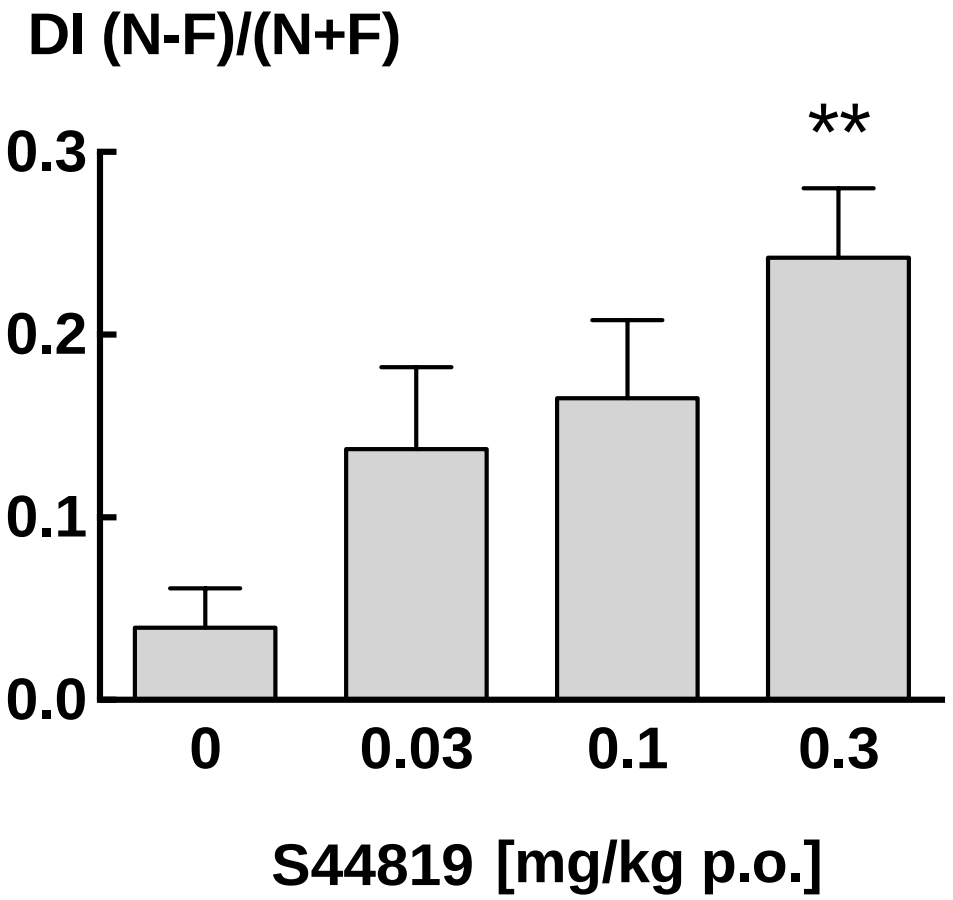


Figure 3

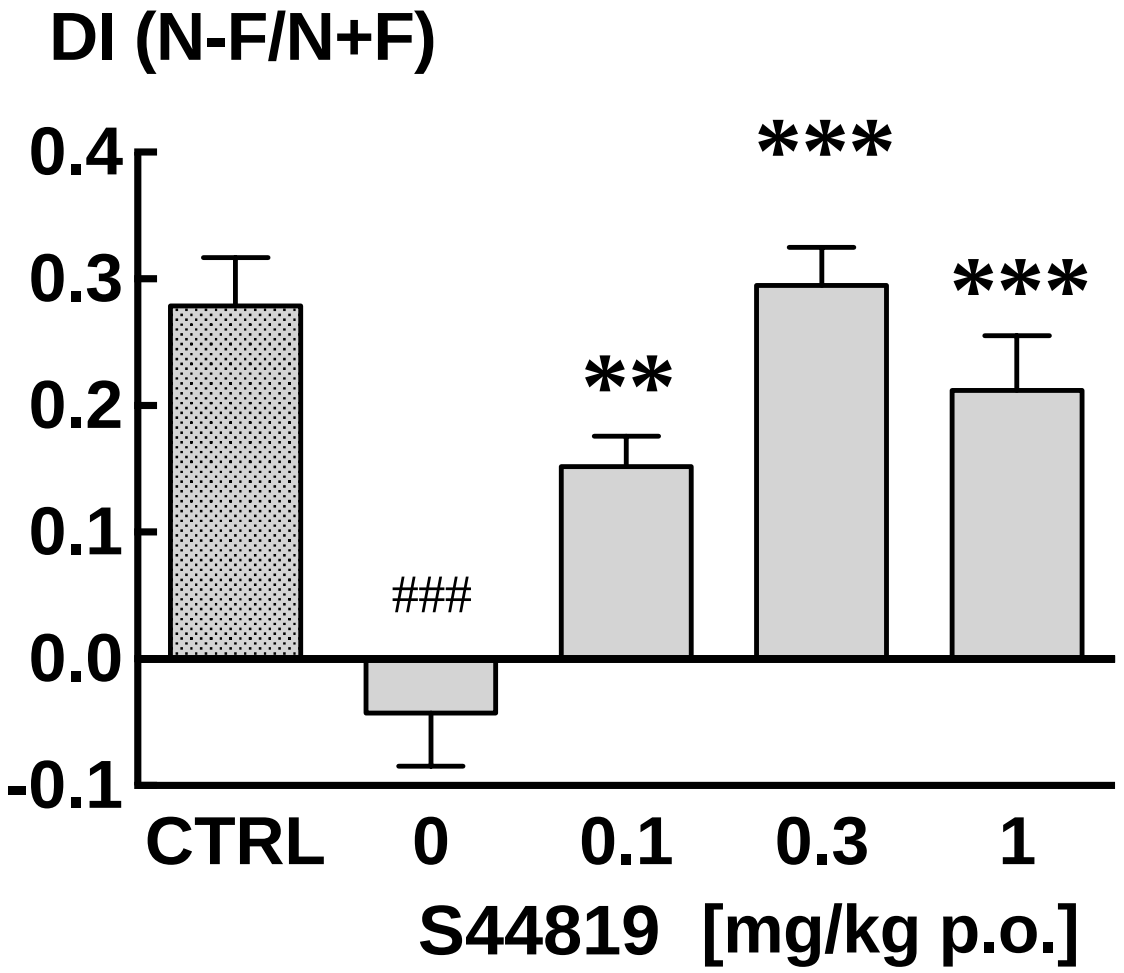
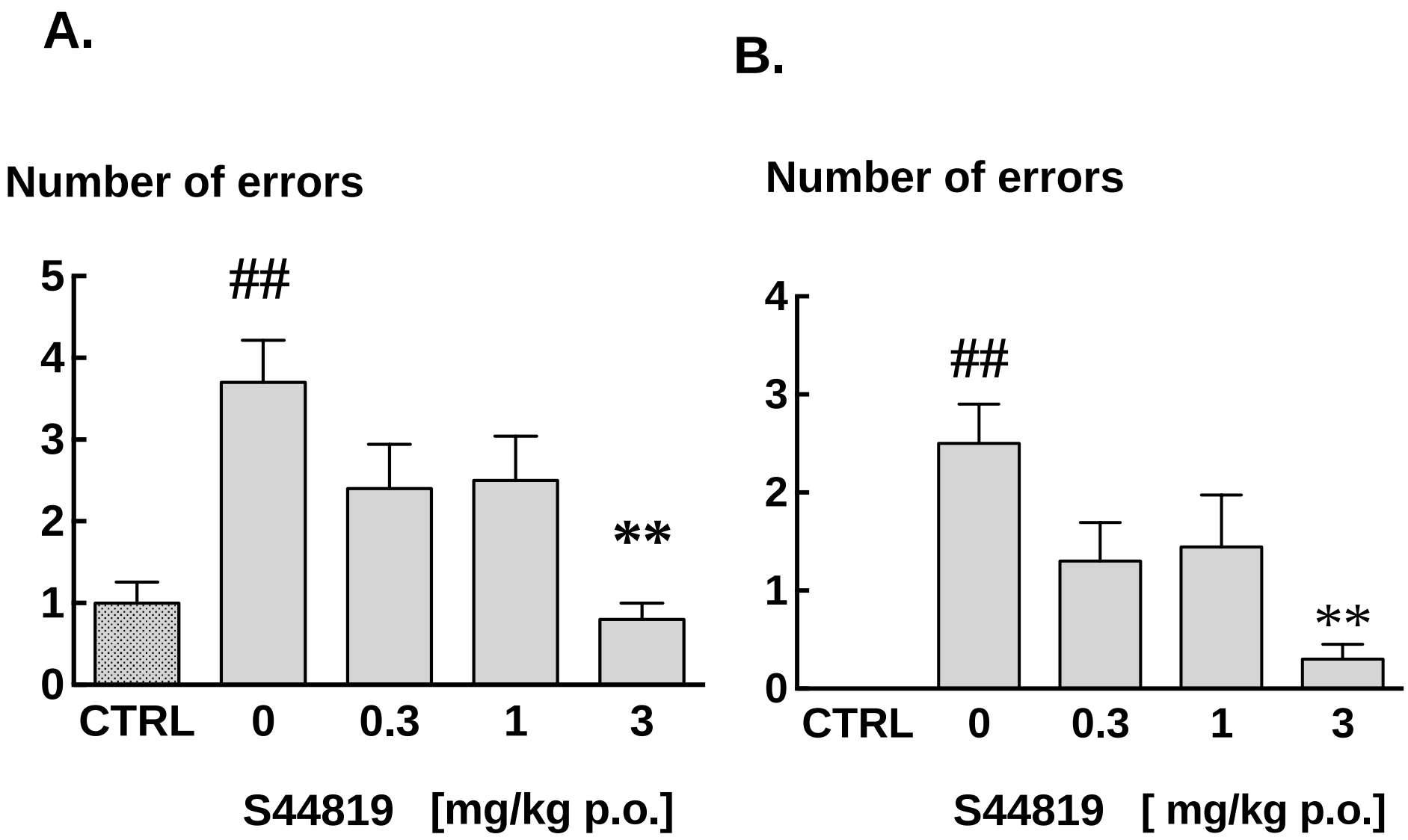


Figure 4


A.
Number of errors
5
4
3
2
1
0
##
**
CTRL
0
0.3
1
3
S44819 [mg/kg p.o.]
B.
Number of errors
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3
2
1
0
##
**
CTRL
0
0.3
1
3
S44819 [mg/kg p.o.]

Figure 5

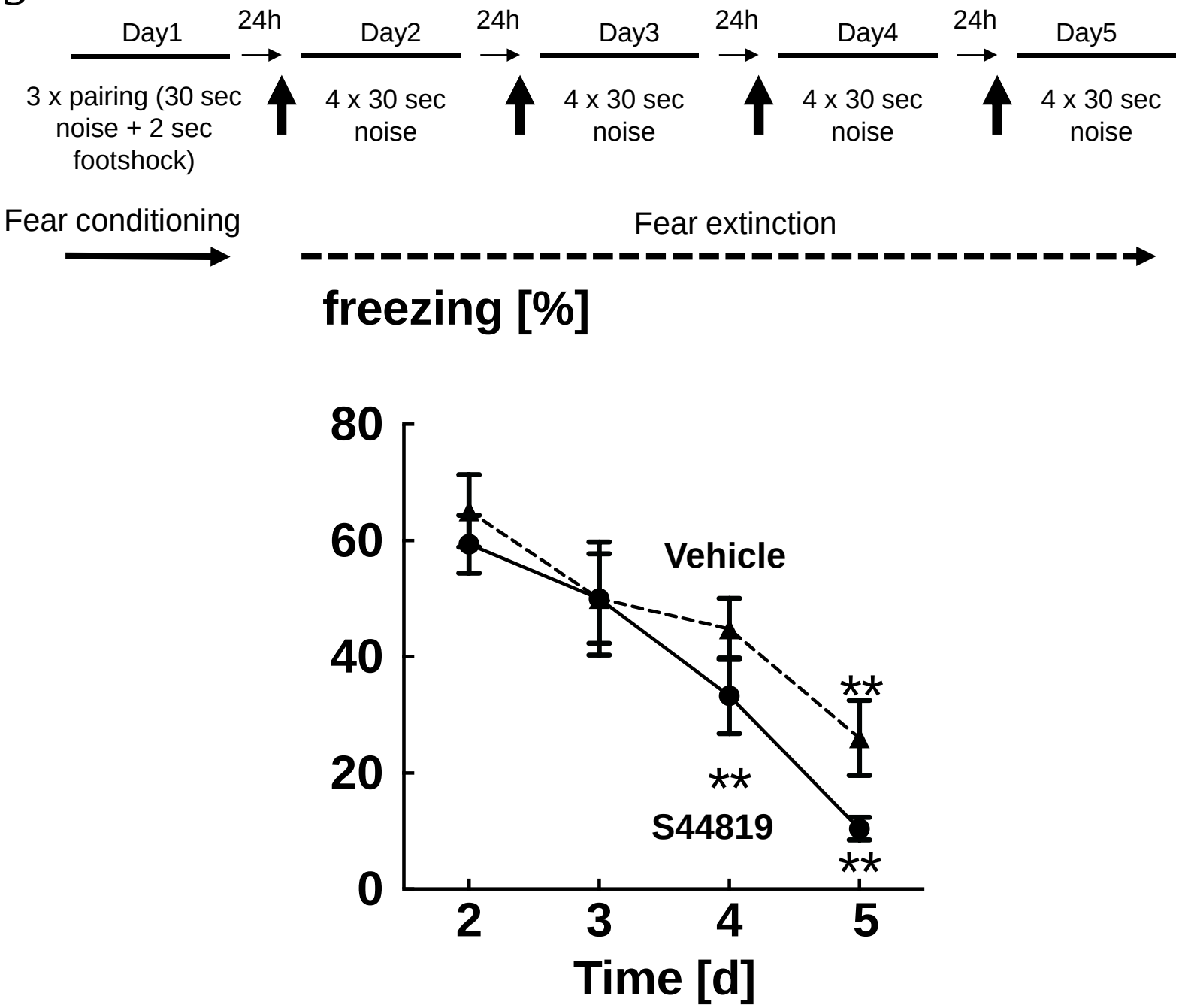

Day1
24h
Day2
24h
Day3
24h
Day4
24h
Day5
3 x pairing (30 sec noise + 2 sec footshock)
4 x 30 sec noise
4 x 30 sec noise
4 x 30 sec noise
4 x 30 sec noise
Fear conditioning
Fear extinction
freezing [%]
80
60
40
20
0
Vehicle
S44819
**
**
**
2
3
4
5
Time [d]

Figure 6

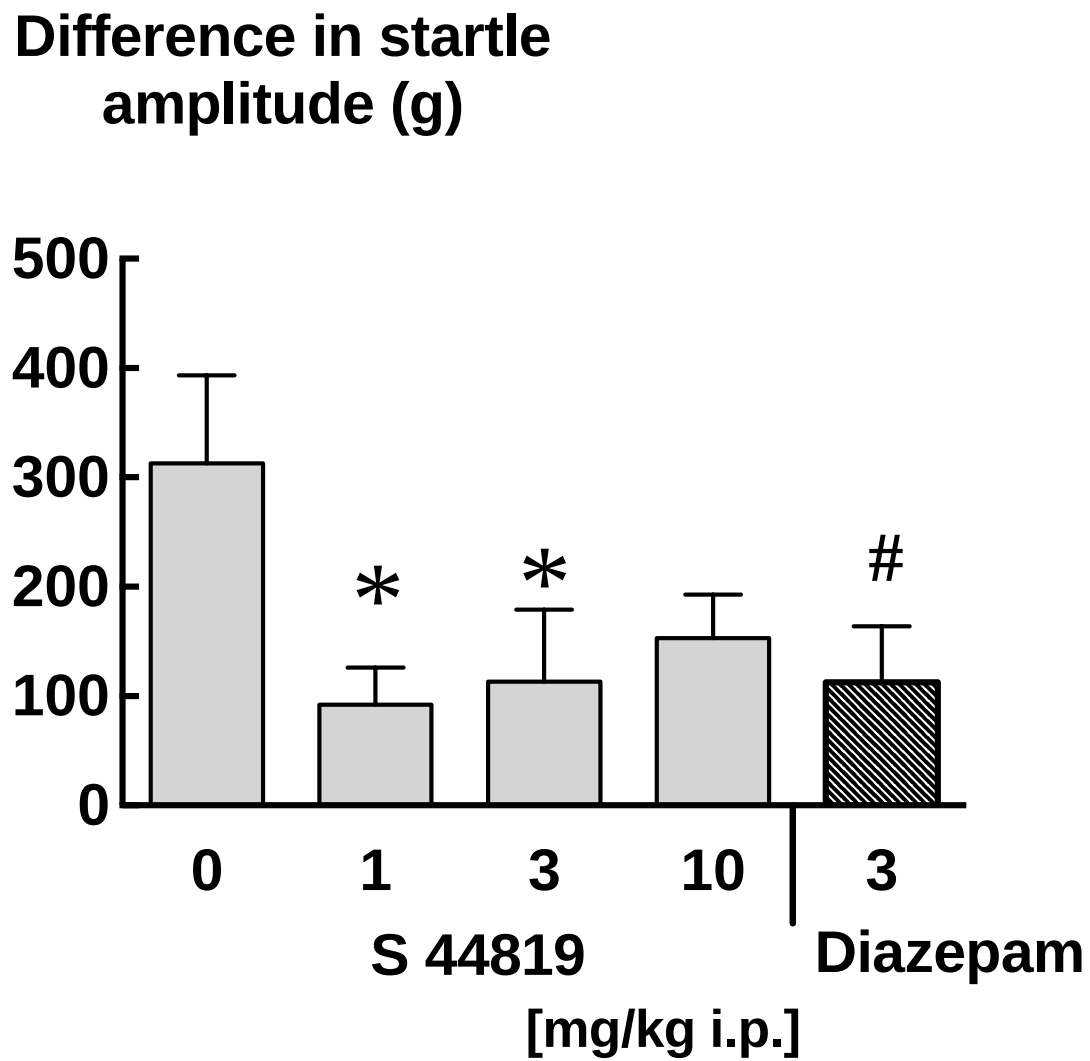

Difference in startle amplitude (g)
500
400
300
200
100
0
*
*
#
0
1
3
10
3
S 44819
Diazepam
[mg/kg i.p.]

Figure 7

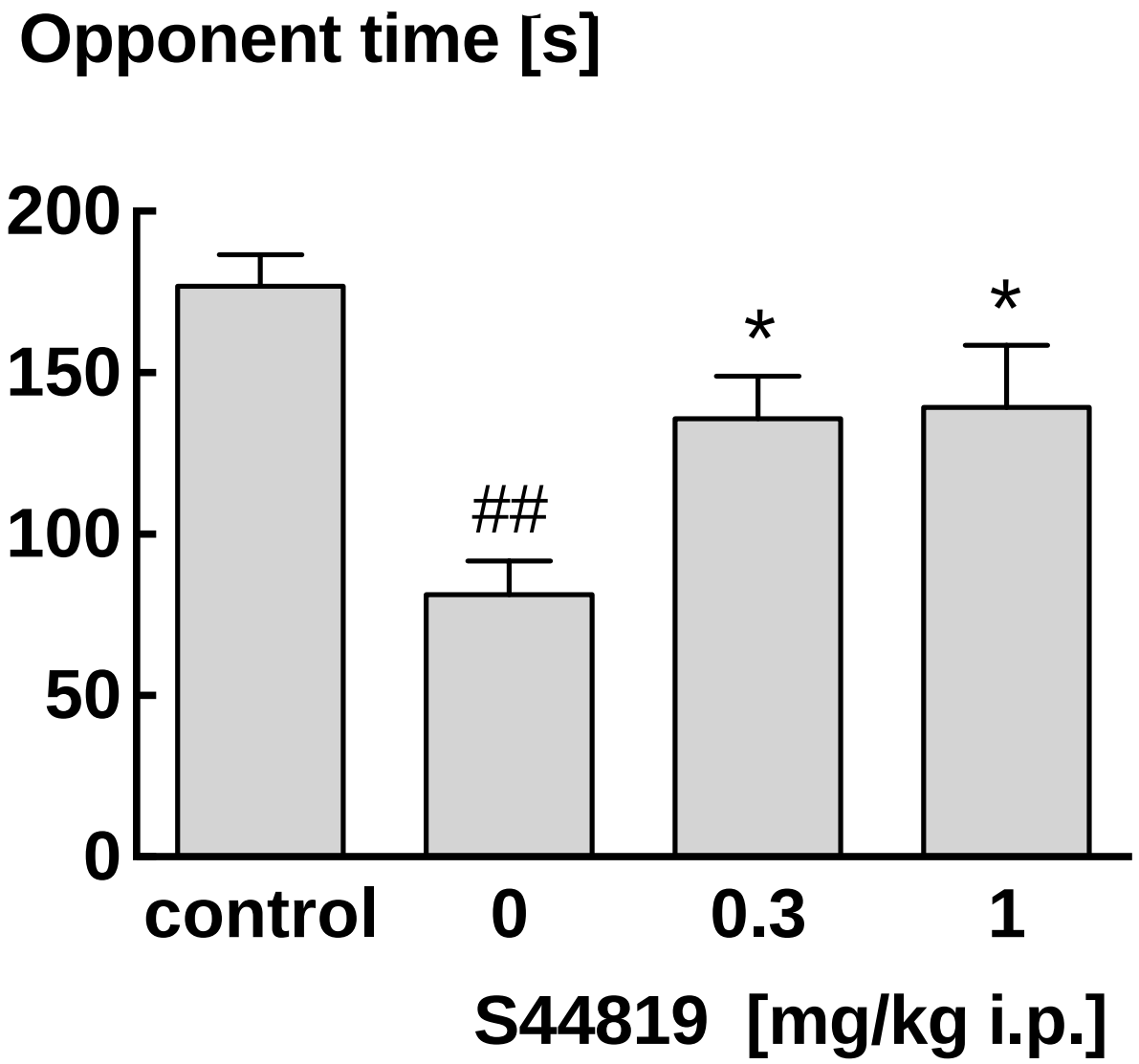
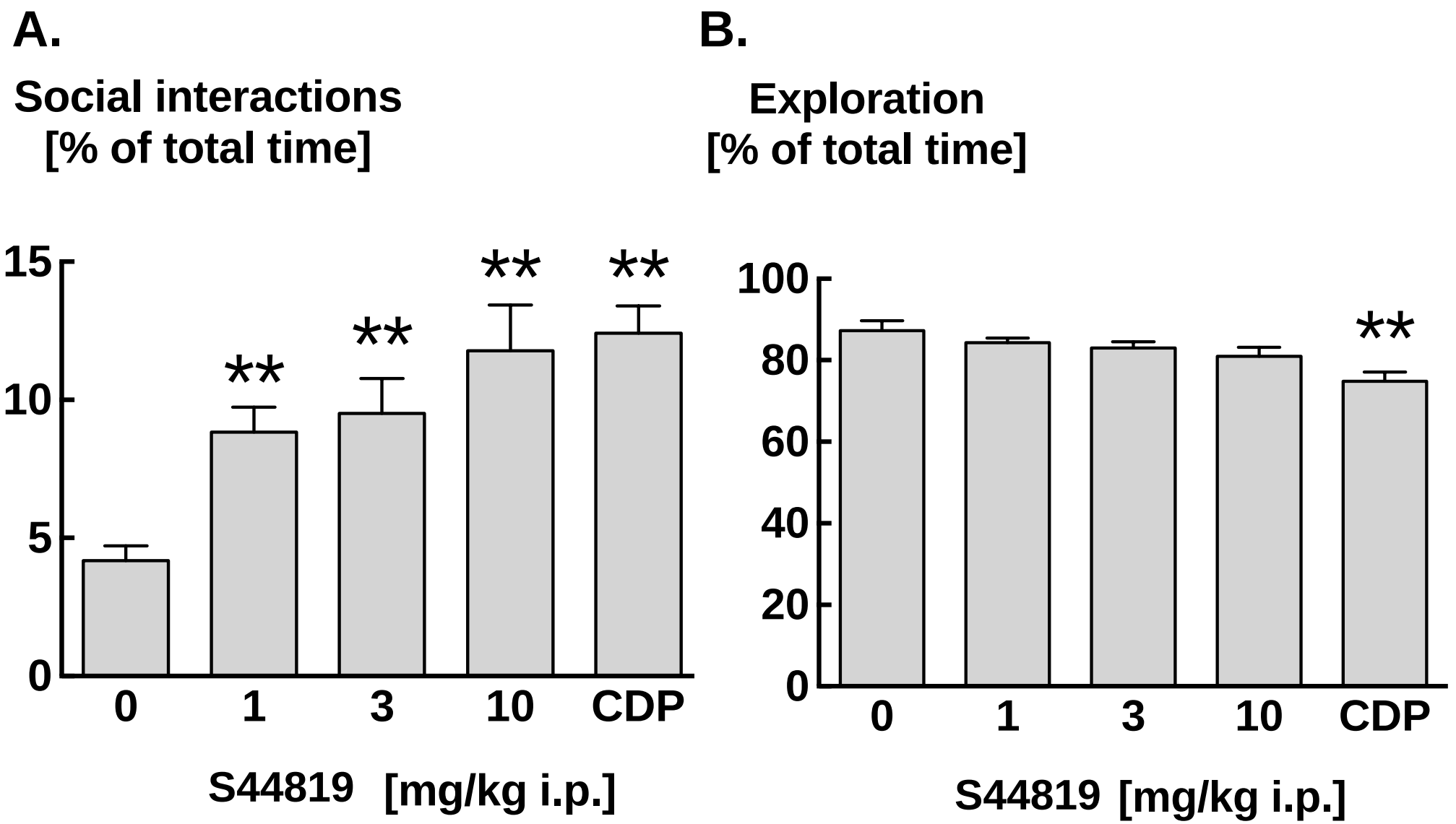


Figure 8


A.
Social interactions
[% of total time]
15
10
5
0
**
**
**
**
0
1
3
10
CDP
S44819 [mg/kg i.p.]
B.
Exploration
[% of total time]
100
80
60
40
20
0
**
0
1
3
10
CDP
S44819 [mg/kg i.p.]

Highlights:

• A novel competitive inhibitor selective for extrasynaptic α5-$GABA_A$ receptors (S44819) was tested in behavioral paradigms.

• The compound was effective in improving long-term as well as short-term (working) memory in mice and rats.

• It was effective in reversing the impairment of object recognition memory induced by sub-chronic treatment of mice with phencyclidine.

• S44819 also showed significant anxiolytic/antidepressant-like activity in tests that have a mnemonic component.

• The compound is now in Phase 2 clinical trials.

Behavioural pharmacology of S 44819: enhancement and remediation of cognitive performance in preclinical models.

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Supplementary information

Materials and Methods

Animals

In experiments male NMRI or C57BL/6J mice (20-30 g) and Male SPRD (Sprague Dawley) rats (270-340 g) were used (Charles River Germany., Egis Pharmaceuticals PLC Hungary). All animals were housed under standard laboratory conditions (24 ± 2 ºC, 40-60 % relative humidity). Animals were kept on a 12-hour light/dark cycle with light onset at 6:00 AM with access to food and water *ad libitum*.

Spontaneous motor activity test in mice (Fig 1A main text)

The experiments were performed according to the method of Borsy et al. (Borsy et al., 1960) in a 10 channel Dews system apparatus, in separate groups of male NMRI mice. The animals were treated intraperitoneally (10 ml/kg) with an acute administration of the vehicle (vehicle group) or three doses of S 44819 (nanoF3, 3, 10 and 30 mg/kg). Sixty min after treatment the mice were placed into the apparatus. Dews system apparatus (10 automated boxes: 44 x 8 x 10 cm) was used in these experiments. The movement of animals was measured with 3 parallel infrared beam interruptions. The number of infrared beam interruptions/channel was recorded for 30 min.

Kindling by chronic administration of drugs (Fig 1B main text)

Kindling is a process by which repeated neuronal activation leads to a long lasting increase in the efficiency of transmission. The resulting hyperexcitability allows a previously ineffective or subthreshold stimulus to provoke seizure activity. Male NMRI mice were used (Charles River Germany., Egis Pharmaceuticals PLC Hungary). The animals were treated with 40

mg/kg *i.p.* of nano F3 S44819 or pentylenetetrazole once daily for 19 consecutive days. On each day, the mice were placed into a transparent colourless glass cylindrical pot (diameter 10 cm, height 12 cm) immediately after drug administration and observed for the next 45 min. During the observation period, the behaviour of the animals (e.g. hypolocomotion, Straub tail, slit eyes and flattened ears) was recorded in addition to the incidence of myoclonic jerks and generalized seizures (characterized by clonic or tonic contraction of the limbs, including loss of righting reflex, i.e., the mice fell onto their side or back). After the 45-min observation period, mice were returned to their home cage. The treatment regime with pentylenetetrazole was previously published (Dawson et al., 2006; Stephens and Turski, 1993), while S 44819 was administered at 40 mg/kg because its biological effects appeared at much lower doses.

Plasma and brain levels of S44819 in C57bl/6 mice (Suppl. Fig 1 and 2)

Adult male C57Bl/6J Harlan (Harlan (Envigo) Laboratories, Horst, The Netherlands) or NMRI (Animal House, Egis Pharmaceutical PLC) BW 20-30 g were studied in the pharmacokinetic experiments. Animals were housed 6 per cage in humidity- (60±10 %) and temperature-controlled (23±1 ^{o}C) rooms with a 12 h light/dark cycle. Food and water were provided *ad libitum*. All dosing suspensions (0.1, 0.3, 1.0 , 3.0 mg/kg) were freshly prepared on the day of testing, and were administered to mice in a volume of 20 ml/kg for *per os* (*p.o.*). Mice were deeply anesthetized with diethyl-ether (Thomasker Finomvegyszer Kft., Budapest, Hungary) and blood samples (0.4-0.7 ml) were taken from the vena cava posterior into Lithium-Heparin tubes (SARSTEDT 1.3ml LH). The subsequent analytical procedures were identical for rat and mouse samples except for appropriate volume adjustments.

Sample preparation

Brains were homogenized with motor driven glass-Teflon homogenizers in distilled water (1:3=w:v). Acetonitrile (HLPC grade, Merck Kft., Budapest, Hungary) (200 µl) was added to 50 µl of brain homogenate or plasma and the mixture was agitated on an IKA Vibrax-VXR platform shaker for 30 min at 1500 r.p.m. at RT, followed by centrifugation for 15 min at 15,000 r.p.m, at RT in an Eppendorf microfuge. One hundred µl of the supernatant were carefully removed and vortex-mixed with 100 µl distilled water. Ten µl of the mixture was

used for LC-MS/MS measurement. Two parallel aliquots of each brain or plasma sample were assayed.

HPLC-MS/MS

Drug levels were determined by HPLC-MS/MS (PAL System, CTC Analytics AG.; HP Series 1200, Agilent and Thermo TSQ Quantum Ultra, Thermo Scientific, supplied by UNICAM, Hungary Ltd.). Analysis was in Focus Mode on a Thermo TurboFlow CycloneP column (50 x 0.5 mm) coupled to a Thermo Hypersil GOLD analytical column (50x3 mm, 5 µm). The loading pump used mobile phases of 10 mM Ammonium acetate (Merck Ltd., Budapest, Hungary) buffer (pH 8.8) [D], distilled water [C] and Acetonitrile (AcCN) (Merck Ltd., Budapest, Hungary) with 0.1 % trifluoro-acetic acid (TFA) (UvaSol, Merck Kft. Budapest, Hungary) [B]. The eluting pump used mobile phases of 10 mM Ammonium formate (MicroSelect, Fluka Chemie Ag.) buffer with 5 % AcCN (Merck Ltd., Budapest, Hungary) and 0.1 % TFA [A] and AcCN with 0.1 % TFA [B]. The LC separation protocol is shown in Table 1. Detector: MS/MS detection was via positive electrospray and MRM mode of scanning. Sample tray temperature: 23 °C; injection volume: 10 µl and the total run time was 7.10 min.

TABLE 1

Step	Start	Sec	Flow	Grad	%A	%B	%C	%D	Tee	Loop	Flow	Grad	%A	%B
1	0:00	30	1.5	Step	-		-	100	----	out	0.65	Step	100	
2	0:30	60	0.2	Step	-		-	100	T	in	0.55	Step	100	
3	1:30	30	1.5	Step			100			out	0.40	Step	100	
4	2:00	30	1.5	Step	-	100	-		----	out	0.40	Ramp	35	65
5	2:30	60	1.5	Step	-	100	-		----	out	0.40	Ramp	5	95
6	3:30	30	1.5	Step	-	100	-		----	in	0.40	Step		100
7	4:00	40	1.5	Step	-	100	-		----	in	0.40	Step		100

8	4:40	30	1.5	Step			100			out	0.40	Step		100
9	5:10	60	1.5	Step	-		-	100	----	out	0.40	Step		100
10	6:10	60	1.5	Step	-		-	100	----	out	0.65	Step	100	

Two parallel runs of calibration samples were also prepared (known concentrations of the drugs spiked into blank plasma and homogenized brain, the whole sample preparation process was performed). Blank plasma and brain samples were injected before the calibrations for the system suitability test. The chromatograms of blank plasma/brain samples had been free of interference for the quantitation. Peak area at the retention interval of the target analyte did not exceed 25 % of the LLOQ (Lower Limit Of Quantitation) peak area. The signal to noise ratio was $\geq$5:1 for the analyte peak at LLOQ level. Calibration curves were generated and analysed as the manufacturer's recommendation using Thermo Xcalibur LC Quan software using 1/x as weighing factor. Calibration standards at the LLOQ level were not allowed to differ no more than 20% of nominal concentration, all other points in the calibration were allowed to differ less than 15%.

Inclined screen test (Suppl. Fig. 3)

This test measures muscle tone, strength, and balance and was carried out in male NMRI mice. S 44819 (Nano F3; 3-100 mg/kg) or the reference compounds diazepam or baclofen (both 10-100 mg/kg) were administered acutely (in a volume of 10 ml/kg *i.p.*). Thirty min later each mouse was placed on a 33 cm x 52 cm, 1 cm x 1 cm square wire mesh panel that was tilted 60° to the horizontal plane of the floor. Five mice were tested simultaneously. ED_{50} was estimated by plotting log dose *vs.* the percentage of animals that had shown signs of muscle weakness (slipped or fell of the screen) during an observation period of 30 s from a total of 10 mice receiving the same dose.

Vogel test in rats (Suppl. Fig. 4)

The test was as originally described by Vogel et al. (Vogel et al., 1971; Volk et al., 2011). Male SPRD rats were water-deprived for 24 h prior to being placed in the experimental

chambers for 1 min and allowed to drink from the water-spout (habituation session). The animals were water-deprived and fasted for another 24 h. Following this second deprivation period, rats were treated either with vehicle solution or three doses of S44819 ((nanoF3; 1 , 3 and 10 mg/kg) acutely (in a volume of 2 ml/kg *i.p.*). Sixty min after treatment, animals were again placed in the chambers for 5.5 min for a test session, where after a 30 s grace period every completion of 20 consecutive licks on the water spout was punished by a 0.6 mA shock (0.6 s) to the tongue during the 5 min. The plastic chambers (19 x 19 x 17 cm), with metal grid floors were connected to a shock generator delivering an electric shock to the water spout located in the center of one of the walls 5 cm above the grid floor. Eight experimental chambers were connected to an IBM PC computer via a central unit (Experimetria Inc, Hungary). The number of licks and tolerated shocks were simultaneously recorded and stored in the computer. The number of tolerated shocks was evaluated. Diazepam (3 and 10 mg/kg) was used as positive control.

Light-dark test in mice (Suppl. Fig. 5)

The male NMRI mice were placed in the test room for a day before the testing and they were deprived of food for approximately 12 h (Kapus et al., 2008; Volk et al., 2011). After intraperitoneal treatment (10 ml/kg), animals were kept in a cage during the pre-treatment time. After 30 min (Diazepam 3 mg/kg i.p.) or 1 h (S44819 nanoF3; 30 mg/kg *i.p*) pre-treatment time, the treated animals were placed into the light-dark apparatus. The light-dark apparatus consisted of 7 Plexiglas cages (41 x 41 x 30 cm) divided into two compartments. The open compartment was open at the top made of transparent Plexiglas and brightly illuminated by an 85 W (Osram) tungsten bulb. The dark compartment was made of black Plexiglas and was covered at the top. A small opening connected the two compartments. Activity was measured automatically by interruptions of horizontal and vertical infrared beams (Omnitech Animal Activity Monitor, *Omnitech* Electronics Inc., Columbus, Ohio, USA). Mice were individually placed in the centre of the open compartment, facing away from the partition and allowed to explore the apparatus for 5 min. The horizontal activity and movement time in the light compartment were evaluated.

Elevated plus maze test in mice (Suppl. Fig. 6)

The test was adapted from the method of Lister (Kapus et al., 2008; Lister, 1987). Male NMRI mice were treated acutely with vehicle (vehicle group) or three doses of S44819 (nanoF3; 3, 10 and 30mg/kg) or the reference compound Diazepam (3 mg/kg)) (in a volume of 10 ml/kg, *i.p.*). Sixty min after treatment the mice were placed in the central area of the plus maze. Animals were individually tested in the plus-maze. The plus maze had a matte black acrylic surface, and consisted of four arms (two open without walls and two enclosed by 15 cm high walls) each arm was 30 cm long and 5 cm wide and fixed-up 60 cm above floor. The behaviour of the animals in the maze was recorded by a video camera in an adjacent room for 5 min, and evaluated with the TSE VideoMot2 software (TSE Systems GmbH, Bad Homburg, Germany). The means ± S.E.M. of time spent by each mouse in the open arms and those of the number of open arm entries calculated in each group.

Marble burying test in mice (Suppl. Fig. 7)

Four doses of S 44819 (Aqoat; 1, 3, 10 and 30 mg/kg) or vehicle were administered orally to male NMRI mice in a volume of 20 ml/kg body weight. After 120 min the mice were placed individually into the experimental chamber which was a 22 x 16 x 14 cm Acrylic box with 5 cm thick layer of sawdust on the bottom. Twenty-four glass marbles (16 mm in diameter) were placed in close contact on the sawdust in the middle of the cage. The mice were left in the cage with the marbles for 15 min after which they were removed and the number of marbles, that were covered more than two thirds by sawdust (i.e. buried), was counted.

Forced swim test (Porsolt test) in mice (Suppl. Fig. 8)

The test was conducted using a modification of the method of Porsolt et al. (Porsolt, 1981; Volk et al., 2011). Three doses of S 44819 (Aqoat; 3, 10 and 30 mg/kg), vehicle or reference venlafaxine (30 mg/kg) were administered orally to male C57BL/6J mice at a volume of 20 ml/kg body weight. After 120 min the mice were placed individually into glass cylinders which

were 25 cm high, 10 cm in diameter and contained 12 cm high water. The temperature of the water was 25 ^{o}C. Four mice were tested simultaneously. Duration of immobility was measured during the last 4 min of the 6 min testing period.

Tail suspension test in mice (Suppl. Fig. 9)

Automated TST device (TSE, Tail suspension System V 2.2, TSE System GmbH, Bad Homburg, Germany) was used to measure the total sum of periods of immobility and the force of movement of the mice. Three doses of S 44819 (S 44819 (Aqoat; 3, 3 and 10 mg/kg),) or vehicle or reference venlafaxine were administered orally to the male C57BL/6J mice at a volume of 20 ml/kg body weight. Two hours later the mice were suspended by their tails using adhesive scotch tape, to a hook connected to a strain gauge (in a separate measuring box) that detected the movements of the mice and transmitted them to a central unit that calculated the total duration of immobility and amplitude (%) during a 6 min test. The maximal force provided by the apparatus is 300 pounds. The amplitude is the force produced by the mice which is expressed as % of maximal force. Mice were considered immobile if the force under 2.25 pounds measured by the apparatus. The sums of immobility time were evaluated by groups.

Pentylenetetrazole-induced seizure threshold test in mice (Suppl. Fig. 10)

The experiments were performed according to the method of Dawson et al. (Dawson et al., 2006; Ling et al., 2015). Male NMRI mice were treated acutely with vehicle (vehicle group) or with three doses of S44819 (Nano F3; 3, 10 and 30 mg/kg) intraperitoneally (10 ml/kg) Thirty min after intraperitoneal administration, the mice were infused with pentylenetetrazole (PTZ) intravenously (i.v., 10 mg/ml solution, infusion rate 12 ml/h). A cannula attached to a 2 ml syringe prefilled with PTZ solution was used. For the purpose of infusion, the animal was restrained and needle was inserted into the tail vein. The accuracy of needle placement in the vein was confirmed by appearance of blood in the cannula. The animal was kept in a transparent Perspex box with holes for ventilation. The syringe was held in the adjustable motor driven infusion pump (Perfusor fm B. Braun Melsungen AG, Melsungen, Germany). In this way animal could move freely in the box without strain on the attached cannula with no

severe struggling. PTZ was infused at a constant rate of 0.2 ml/min. During the infusion, mice were observed for the onset of different types of seizures by a trained observer who was blind to the treatment regimes. The time latencies from start of infusion to the appearance of first clonus (characterized by the rapid involuntary rhythmic contraction and relaxation of limbs) were recorded. Infusion was stopped at the appearance of clonic seizure in each animal and the corresponding administered dose of PTZ was calculated. The non-selective $GABA_A$ negative allosteric modulator FG7142 (30 mg/kg, *i.p.*) was used as the reference compound.

Statistical analysis

The results were expressed as mean ± S.E.M. The statistical analysis was used with the GraphPad Prism 6.0 program (GraphPad Software, San Diego, USA). The data were analyzed by one-way analysis of variance (ANOVA) followed by a Dunnett's *post-hoc* test for multiple comparisons. The test was validated by Student's unpaired t-test between the control group and the group treated with the respective reference compound. $P < 0.05$ was considered significant.

References

Borsy, J., Csányi, E., Lázár, I., 1960. A method of assaying tranquilizing drugs based on the inhibition of orientational hypermotility. Arch Int Pharmacodyn Ther 124, 180-190.

Dawson, G. R., Maubach, K. A., Collinson, N., Cobain, M., Everitt, B. J., MacLeod, A. M., Choudhury, H. I., McDonald, L. M., Pillai, G., Rycroft, W., Smith, A. J., Sternfeld, F., Tattersall, F. D., Wafford, K. A., Reynolds, D. S., Seabrook, G. R., Atack, J. R., 2006. An inverse agonist selective for alpha5 subunit-containing GABAA receptors enhances cognition. J Pharmacol Exp Ther 316, 1335-1345.

Kapus, G. L., Gacsályi, I., Végh, M., Kompagne, H., Hegedűs, E., Leveleki, C., Hársing Jr., L. G., Barkóczy, J., Bilkei-Gorzó, A., Lévay, G., 2008. Antagonism of AMPA receptors produces anxiolytic-like behavior in rodents: effects of GYKI 52466 and its novel analogues. Psychopharmacology (Berl) 198, 231-241.

Ling, I., Mihalik, B., Etherington, L. A., Kapus, G., Pálvölgyi, A., Gigler, G., Kertész, S., Gaál, A., Pallagi, K., Kiricsi, P., Szabó, E., Szénási, G., Papp, L., Hársing, L. G., Lévay, G., Spedding, M., Lambert, J. J., Belelli, D., Barkóczy, J., Volk, B., Simig, G., Gacsályi, I., Antoni, F. A., 2015. A novel GABA(A) alpha 5 receptor inhibitor with therapeutic potential. Eur J Pharmacol 764, 497-507.

Lister, R. G., 1987. The use of a plus-maze to measure anxiety in the mouse. Psychopharmacology (Berl) 92, 180-185.

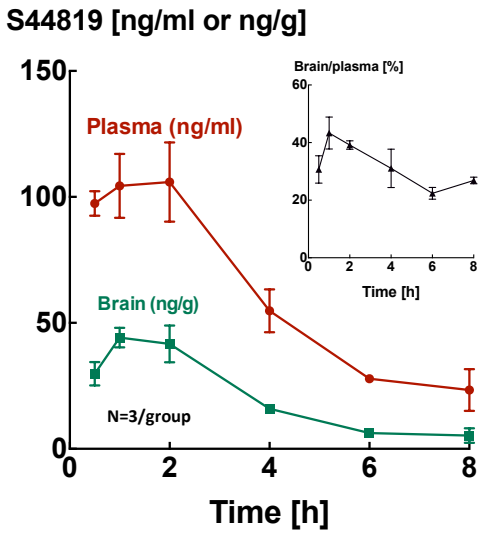
Porsolt, R. D., 1981. Behavioral dispair. In: Enna, S. J., Malick, J. B., Richelson, E., (Eds), Antidepressants:Neurochemicsl, Behavioral , and Clinical Perspectives. Raven Press, New York, pp. 121-139.

Stephens, D. N., Turski, L., 1993. Kindling to the benzodiazepine receptor inverse agonist, FG 7142: evidence for involvement of NMDA, but not non-NMDA, glutamatergic receptors. Neuropharmacology 32, 1011-1017.

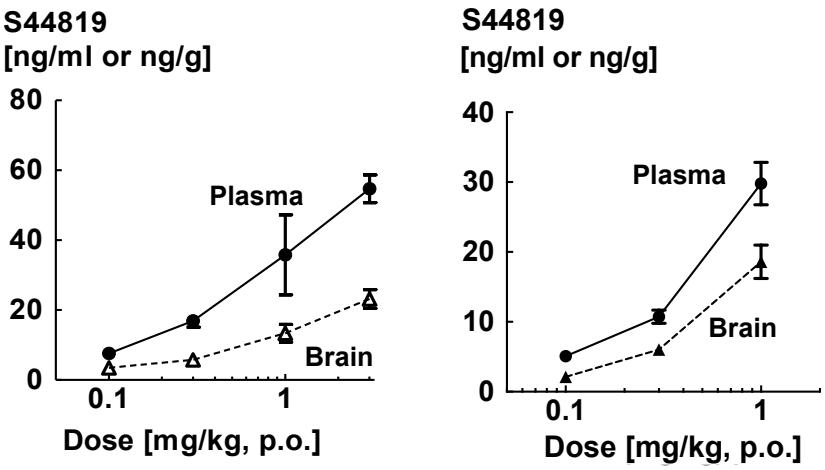
Vogel, J. R., Beer, B., Clody, D. E., 1971. A simple and reliable conflict procedure for testing anti-anxiety agents. Psychopharmacologia 21, 1-7.

Volk, B., Gacsályi, I., Pallagi, K., Poszavácz, L., Gyönös, I., Szabó, E., Bakó, T., Spedding, M., Simig, G., Szénási, G., 2011. Optimization of (arylpiperazinylbutyl)oxindoles exhibiting selective 5-HT(7) receptor antagonist activity. J Med Chem 54, 6657-6669.

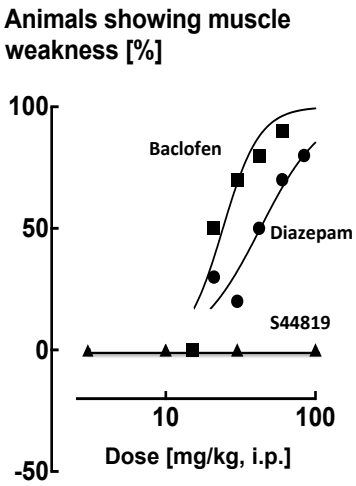
Supplementary FIGURES



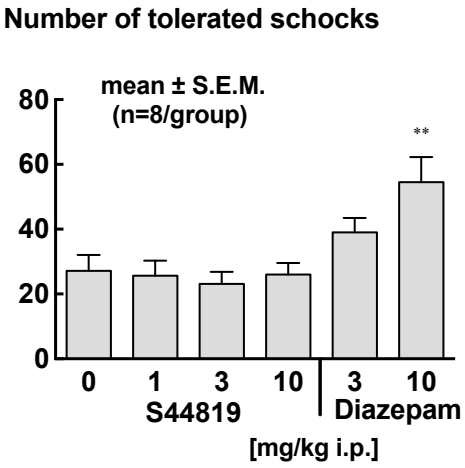
Suppl. Fig. 1 *Time-course of the concentrations of S44819 in plasma and brain homogenates in male C57Bl/6 mice given 3 mg/kg p.o. S44819 Aqoat at time 0.* The insert shows the levels of S44819 in brain homogenates as a percentage of the plasma concentration from the same animals. Results in C57BL/6J and NMRI mice were closely similar. Data are mean ± S.E.M. , n=3/point. A non-GLP study.



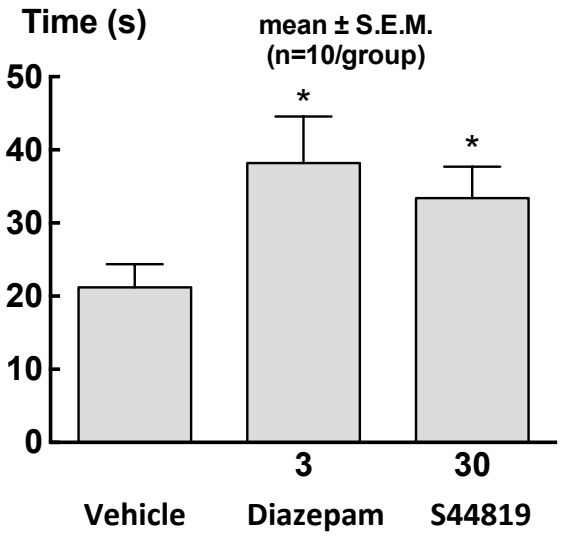
Suppl. Fig. 2 *Concentrations of S44819 in blood plasma and brain of NMRI mice upon administration of various doses of formulated S 44918 p.o.* A) S 44819 nanoF3 at 60 min, B) S44819 Aqoat at 120 min. Data are mean ± S.E.M., n=3/point in A and n=6/point in B.



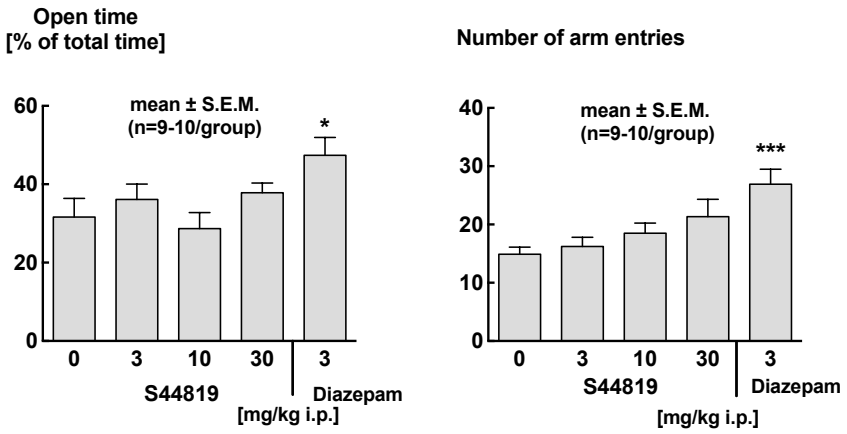
Suppl. Fig.3 *Effect of S44819 in the inclined screen in male NMRI mice.* Single points constructed from the observation of 10 mice per group are shown. S44819 (nano F3), baclofen and diazepam were given *i.p.* 30 min before the start of the test. Curves were fitted by constraining the minimum at 0 % and the maximum at 100 % with GraphPad Prism v6.0 four-parameter non-linear regression. Data are mean ± S.E.M., Single points constructed from the observation of 10 mice per group are shown.



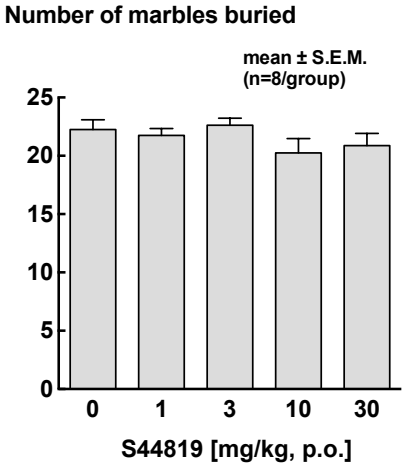
Suppl. Fig. 4 *Effect of S44819 in the Vogel conflict test in male SPRD rats.* Data are mean ± S.E.M., n=8/group. Vehicle, S44819 (nano F3) and diazepam were given *i.p.* 60 min before the start of the test. ** P<0.01 *vs* vehicle, One-way ANOVA followed by Dunnett's test.



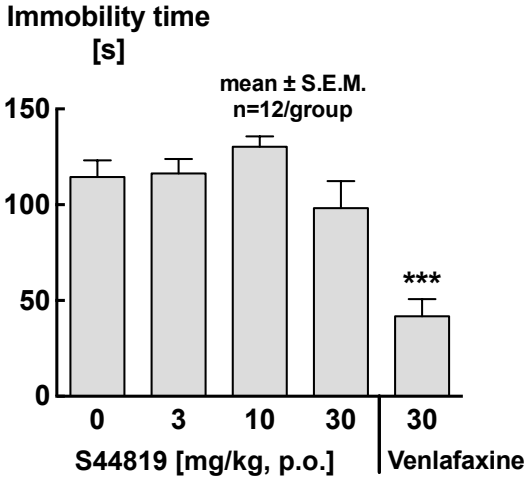
Suppl. Fig. 5 *Effect of S44819 in the light-dark box paradigm in male NMRI mice.* Data are mean ± S.E.M., n=10/group. Vehicle, S44819 (nano F3) and diazepam were given *i.p.* 60 min before the start of the test, diazepam was given *i.p.* 30 min before the start of the test. * P<0.05, One-way ANOVA followed by Dunnett's test.



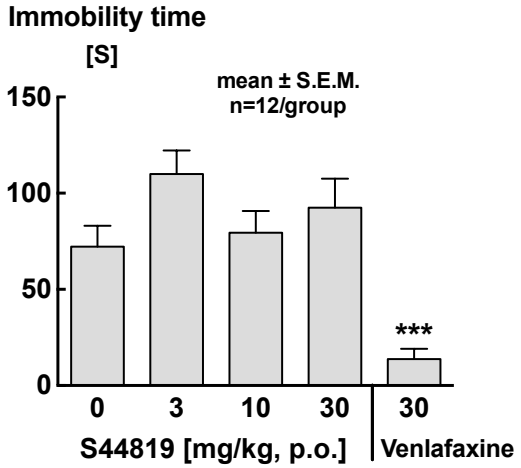
Suppl. Fig. 6 *Effect of 44819 in the elevated plus maze in male NMRI mice.* Data are mean ± S.E.M., n=9-10/group. Vehicle, S44819 (nano F3) were given *i.p.* 60 min before the start of the test, diazepam was given *i.p*. 30 min before the start of the test. * P<0.05, *** P<0.001 *vs* vehicle, Student's t-test.



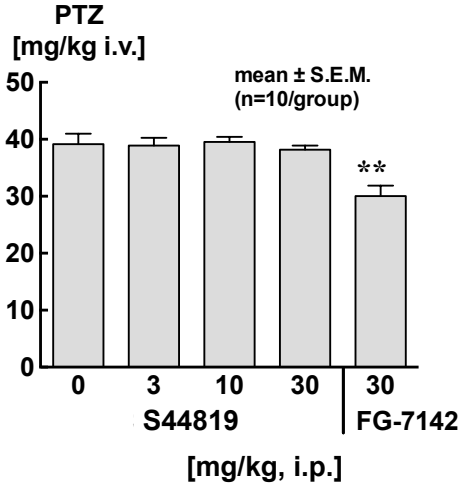
Suppl. Fig. 7 *Effect of S44819 on marble burying activity in male NMRI mice.* Data are mean ± S.E.M., n=8/group. Vehicle or S44819 (Aqoat) were given *p.o.* 120 min before the start of the test. No significant effect one-way ANOVA.



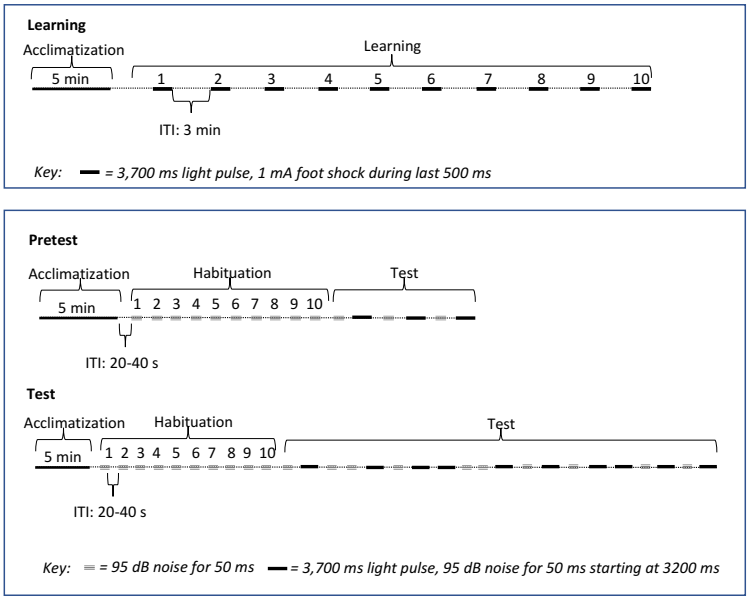
Suppl. Fig. 8 *Effect of S44819 in the Porsolt test (forced swimming) in male C57BL/6J mice.* Data are mean ± S.E.M., n=12/group. Vehicle, S44819 (Aqoat) and venlafaxine were given *p.o.* 120 min before the start of the test, ***P<0.001 vs. control, Student's t-test), No significant effect of S44819 (one-way ANOVA).



Suppl. Fig.9 *Effect of S44819 in the tail suspension test in male C57BL/6J mice.* Data are mean ± S.E.M., n=12/group. Vehicle, S44819 (Aqoat) and venlafaxine were given *p.o.* 120 min before the start of the test. ***P<0.001 vs. control, Student's t-test), No significant effect of S44819 (one-way ANOVA).



Suppl. Fig. 10 *Effect of S 44819 on the pentylenetetrazole (PTZ) convulsion threshold.* Data are mean ± S.E.M., n=10/group. Vehicle, S44819 (nano F3) and FG-7142 were given i.p. 60 min before the start of the intravenous infusion of pentylenetetrazol. ** P<0.01 *vs.* vehicle, Student's t-test. No significant effect of S44819 (one-way ANOVA).



Suppl. Fig.11 *Diagrammatic scheme of the experimental protocol of the fear-potentiated startle response test.*